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# Mechanisms of Benzyl Alcohol Tolerance in *Drosophila melanogaster*

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# Mechanisms of Benzyl Alcohol Tolerance in *Drosophila melanogaster*

by

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## Dedication

I dedicate this thesis to my parents Mahmoud and Nasma Al-Hasan,

my brother Motaz, my sisters Lama and Dana and my partner Julie Owen.

## Acknowledgments

I wish to thank my mentor and teacher Dr. Nigel Atkinson, who brought out the best of my creative, critical and independent thought. His curiosity, insight, patience and willingness to tackle the most difficult of problems, has placed us in a position to handle the diverse questions in neuroscience. I also thank Dr. Rudi Bohm, the graduate student who initiated me into the field of Drosophila neurobiology. I send a special thanks to my labmates Dr. Alfredo Ghezzi and Dr. Harish Krishnan, whose scientific partnership and sincere friendship made my journey through graduate school both productive and enjoyable. I would also like to thank my love, Julie Owen, for being a source of happiness, inspiration and support in all aspects of my life. I thank the remainder of the past and present Atkinson lab members, Jascha Pohl, Dr. Yan Wang, Xiaolei Li, Rosie Ramazani, Brooks Robinson, Ben Troutwine, Dr. Roshani Cowmeadow and Dr. Joyce Yu.

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**Mechanisms of Benzyl Alcohol Tolerance in *Drosophila melanogaster***

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The University of Texas at Austin, 2009

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Proper neuronal function requires the preservation of appropriate neural excitability. An adaptive increase in neural excitability after exposure to agents that depress neuronal signaling blunts the sedative drug effects upon subsequent drug exposure. This adaptive response to drug exposure leads to changes in drug induced behaviors such as tolerance, withdrawal and addiction. Here I use *Drosophila melanogaster* to study the cellular and neuronal components which mediate behavioral tolerance to the anesthetic benzyl alcohol. I demonstrate that rapid tolerance to benzyl alcohol is a pharmacodynamic mechanism independent of drug metabolism. Furthermore, tolerance is a cell autonomous response which occurs in the absence of neural signaling. Using genetic and pharmacological manipulations I find the synapse to play an important role in the development of tolerance. In addition, the neural circuits that regulate arousal and sleep also alter benzyl alcohol sensitivity. Beyond previously described transcriptional mechanisms I find a post-translational role of the $Ca2^{+}$-activated $K^{+}$-channel, *slowpoke* in the

development of tolerance. Finally, I explore a form of juvenile onset tolerance, which may have origins that differ from rapid tolerance. The implications of this study go beyond tolerance in *Drosophila melanogaster* to benzyl alcohol and can shed light on human drug tolerance, withdrawal and addiction.

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# Chapter 1: General Introduction

## Sedation is mediated by specific anesthetic-target interaction

Since the advent of anesthetics and alcohols in clinical practice, they have become some of the most essential therapeutic agents. Despite their widespread use, the molecular mechanisms that mediate sedation and the response of the nervous system to anesthetic exposure have remained elusive. The theories regarding the mechanisms of anesthetic action have centered around two opposing view points: specific and nonspecific. At the turn of the last century, two investigators, Meyers and Overton, independently observed that the potency of an anesthetic was correlated to its hydrophobicity as measured by its water-oil partition coefficients. This observation led to the notion that anesthetics interact with the lipid bilayer and dissolve cell membranes, which in turn perturbs the function of crucial but non-specific membrane bound proteins, leading to sedation. The simplicity of this hypothesis is tempting, however, it fails in four basic aspects. The first is that very hydrophobic long chain alcohols do not produce anesthesia. Secondly, stereoisomers of an anesthetic with the same partition coefficient have differing anesthetic potencies. Thirdly, membrane fluidity is not altered at clinically relevant anesthetic concentrations. Finally, non-anesthetic induced changes in membrane fluidity such as those caused by increasing temperature do not cause anesthesia [47]. The exclusion of non-specific anesthetic/lipid interactions as

the major cause of sedation has led investigators to search for specific protein anesthetic targets. Of these targets the ones that mediate neuronal signaling are most relevant to sedation [22].

Anesthetic action leads to a reduction in over all neuronal electrical excitability [29]. Thus, sedation is ultimately achieved by the interaction of the anesthetic with the molecules that regulate synaptic excitability such as ligand- and voltage-gated ion channels, and the vesicle-release machinery [30]. The ligand-gated receptors which have emerged as strong candidates of anesthetic targets include $GABA_A$, glycine, 5-HT, ACh, NMDA and AMPA. Voltage-gated ion channels including certain $Na^+$ and $Ca^{2+}$ channels have also been identified as possible anesthetic targets. Furthermore, anesthetics are also thought to inhibit vesicle release by directly interacting with at least one member of the vesicle-release machinery, syntaxin1A [31]. Over all anesthetics are “dirty” drugs in the sense that they affect many targets, however, the emerging evidence demonstrates that they do so specifically.

**Anesthetic tolerance is caused by increase in neuronal activity**

The complexity of anesthetic exposure includes not only the mechanism of action but also the reaction of the nervous system to sedation. In clinical situations where long-term anesthetic administration is required, health care providers have noticed the development of tolerance to the sedative affects of anesthesia [4]. The tolerance observed in this setting is exemplified by the requirement of larger doses of sedative to maintain the state of anesthesia. Tobias, J. D. (2000) presents an example of tolerance that develops over a five day intubation of a 9 year old child with trisomy 21 [85]. A plot of the presented data in Tobias, J. D. (2000) demonstrates the development of tolerance over the course of a five day intubation (Figure 1.1).

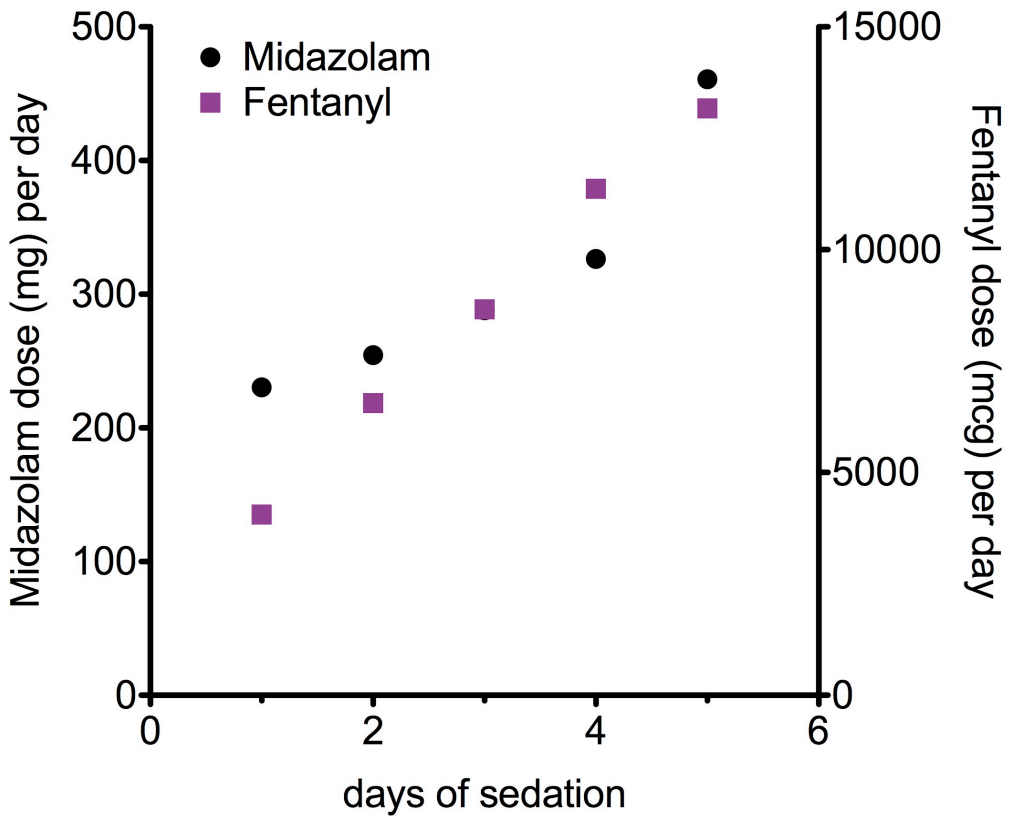



Figure 1.1 Clinical manifestation of tolerance. This is a plot of amount of anesthetic required to sedate a nine year old child with trisomy 21 for five days to facilitate mechanical ventilation. Notice the two fold increase in midazolam and the three-fold increase in fentanyl required to maintain sedation. The data plotted here was originally published by Tobias, J. D. (2000) [85].

Tolerance is defined as a decrease in the effect of a drug over time due to previous exposure to the drug. There are two broad categories of tolerance that are distinguished based on their underlying mechanisms. Pharmacokinetic tolerance arises through an increase in metabolism and clearance or through a decrease in drug absorption. This form of tolerance reduces the affect of a drug by reducing the concentration of the drug at the target tissue. Pharmacodynamic tolerance, a second form of tolerance, is an adaptive response generated in the target tissue to reduce drug efficacy, without decreasing the effective drug concentration. In the context of anesthetics, pharmacodynamic tolerance is a neuro-adaptive response that reduces the sedative effects of exposure by increasing neuronal excitability in a homeostatic manner. This form of plasticity that counters sedation by increasing neural excitability must include changes in ion channel composition, quantity and function and ultimately result in an increase in neurotransmitter release [12]. It has been proposed that this increase in neuronal activity is the underlying cause of some withdrawal symptoms [79]. After discontinuation of the sedative agent, patients often exhibit symptoms of central nervous system activation, which include irritability, insomnia, and seizures, and are relieved by subsequent sedative re-administration [45]. The decrease in seizure threshold and development of tolerance due to anesthetic exposure suggests that the nervous system responds to the sedative affects of anesthesia by an increase in excitability, which causes neurons to fire uncontrollably

resulting in seizure [85]. This thesis will focus on the neuroadaptive mechanisms that underlie the development of tolerance.

**Drosophila as a model organism for the study of anesthetic responses**

Here, I will present experiments conducted on the fruit fly, *Drosophila melanogaster*, which identify some of the *in vivo* mechanisms involved in the development of tolerance to the anesthetic benzyl alcohol. The genetic simplicity and behavioral complexity of the fruit fly has rendered it a pivotal player in the identification and characterization of the molecules that mediate neural function. The Drosophila nervous system consists of more than 100,000 neurons that mediate a spectacular behavioral repertoire. Flies do not only exhibit the basic sensory and motor functions, they can also navigate on land and in the air, exhibit photo and geotaxis, have a finely tuned circadian locomotor and feeding rhythm, possess short and long term memories, exhibit a well defined mating and courtship ritual, and engage in aggressive behaviors. The study of Drosophila behavior led to the cloning of the first voltage-activated $K^+$ channel, *Shaker*, based on a shaking phenotype caused by mutation of the *Shaker* locus at elevated temperatures [82]. Because of the strong homology between genes involved in critical aspects of neuronal function, *Shaker* was subsequently cloned from vertebrates. Because flies contain virtually all the neural components found in vertebrate neurons [72], the genes found to play a role in anesthetic

function in Drosophila have also been identified to play a similar role in vertebrate systems. Mutations in the Drosophila *shaker* locus also alter anesthetic sensitivity [83,93], and this channel is thought to play a similar role in mammalian systems [2].

The structure of the Drosophila anesthetic response is also remarkably similar to the one observed in mammals [26]. This pattern begins with an initial hyper-excitable phase where the animals exhibit an increase in locomotor activity followed by a soporific phase in which they lie motionless on their backs and finally, after drug removal, the animals begin to regain consciousness and display their typical pre-anesthetized climbing behavior. Even though exposure to anesthetics leads to tolerance to any or all of the behavioral changes caused by anesthetic exposure, I chose to focus on recovery from sedation. Of the three phases, it is the slowest, making small changes in behavior during this phase more evident. Recovery from sedation is also temporally far enough away from the other phases that it is not obscured by plasticity in the hyper-active phase or sedation. Furthermore, recovery from sedation is a clearance phase where drug metabolism is occurring without absorption while during the initial hyper-active and sedation phases, absorption and metabolism occur simultaneously.

## Summary of previous findings

The work presented here is a continuation on previous work that others and I have conducted in the past. Our previous findings indicate that tolerance to the anesthetic benzyl alcohol results from a homeostatic mechanism. Homeostasis is a term that describes a biological process that maintains physiological equilibrium. In the context of tolerance to sedative agents that shift neuronal equilibrium towards an inhibited state, a homeostatic response acts to counter the reduction in signaling by increasing neuronal activity. Upon subsequent sedation, this increase in neuronal signaling causes a more rapid recovery as it counteracts the sedative effects of the anesthetic. We have identified this activity-dependent modulation of neuronal activity that leads to tolerance to be dependent on the expression of the $Ca^{2+}$-activated $K^{+}$ channel, *slowpoke*. The induction of this channel after sedation increases neuronal signaling, and thus shifts neuronal activity to a more excited state. Upon subsequent sedation, pre-sedated animals recover more rapidly, hence the development of tolerance [26]. Furthermore, Wang et. al. (2009) found an increase in occupancy of the cAMP Responsive Element Binding Protein (CREB) at the *slowpoke* promoter after benzyl alcohol sedation [91]. This finding suggests that *slowpoke* is just one component of a larger system involved in the development of tolerance.

**Dissertation Overview**

The overall goal of this study is to improve our understanding of the *in vivo* mechanisms involved in the development of behavioral tolerance to the anesthetic benzyl alcohol in the fruit fly, *Drosophila melanogaster*.

Chapter 2 is a study that demonstrates that rapid benzyl alcohol tolerance does not have metabolic origins. In this chapter, I use gas chromatography to quantify the concentration of benzyl alcohol found in flies after administration and as they recover from sedation. After generating a dose-response curve it became evident that at elevated levels of anesthetic, tolerant animals showed a reduction in the resulting behavioral effects compared with naive animals.

Chapter 3 investigates several aspects of rapid benzyl alcohol tolerance. In this chapter, I provide electrophysiological data demonstrating that benzyl alcohol causes a suppression of centrally located neurons. I also demonstrate that rapid tolerance does not arise from a drug independent gross reduction in neuronal signaling. Then I conduct a mutant and pharmacological analysis, in which I show that the synapse is an important site in the development of tolerance. Finally, I demonstrate that tolerance is a cell-autonomous response to drug exposure.

Chapter 4 illustrates the ability to rescue the tolerant defective phenotype of a *slowpoke* mutation with a transgene that expresses *slowpoke* under the control of a heatshock promoter. This result suggests

post translational modifications are involved in the development of tolerance.

Chapter 5 explores the brain regions involved in benzyl alcohol resistance and sensitization. In this chapter, I induce *slowpoke* expression in a subset of neuronal regions and structures and test for changes in benzyl alcohol sensitivity. I found that induction of *slowpoke* within the mushroom and ellipsoid bodies causes resistance to benzyl alcohol sedation, while induction of *slowpoke* in the cells that regulate circadian rhythm causes sensitization.

In chapter 6, I present data on a form of tolerance induced in larvae that persists through metamorphosis. In this chapter, I study the effects of exposure to benzyl alcohol during the larval stages on adult flies, and find that larval exposure leads to tolerance in the adult. My data indicates that this form of tolerance may have different origins than rapid tolerance.

Fruit flies are amenable to behavioral studies making anesthetic endpoints easy to define. In the studies presented here, I use the inability to climb as the anesthetic endpoint, and I score recovery as a return to climbing. The behavioral data presented here will be plotted on x-y plane, where the x-axis is time in minutes and the y-axis is the percent of flies that have began climbing the sides of the vial after sedation with benzyl alcohol.

# Chapter 2: Rapid Benzyl Alcohol Tolerance is Pharmacodynamic

## Introduction

Drug tolerance is defined as a reduction in drug efficacy due to previous exposure to the drug. The mechanisms that underlie the development of tolerance is of great importance to both scientists and clinicians. In an experimental setting, drug tolerance represents an adaptive mechanism set into motion by drug exposure that works to reduce the effects of the drug on subsequent exposures. Clinically, drug tolerance presents a major obstacle in long term therapy, as medications lose their desired effects. There are two mechanisms that underlie the development of tolerance: a reduction in effective drug levels caused by an increase in clearance or a reduction in absorption, and a second form where the target tissue adapts as to become resistant to some or all of the drug effects. A dose-response curve, which relates the concentration of a drug to an effect of the drug, can be used distinguish between tolerance of metabolic origin and functional tolerance. If tolerance arises through a pharmacokinetic mechanism, one would observe a reduction in drug concentration due to an increase in drug clearance or a decrease in drug absorption, and thus the dose-response relationship between the tolerant and naive population should be identical. However, if the tolerant phenotype is caused by a pharmacodynamic mechanism, the effect of the

drug will be reduced while the drug concentration remains constant, leading to a right-ward shift in the dose-response relationship.

In this thesis, I focus on a form of tolerance called rapid tolerance. This type of tolerance occurs in response to a single drug exposure and is extant following drug clearance. We detect it as a diminished response to a subsequent drug administration. This thesis is centered around the mechanisms that underlie the development of rapid tolerance to the sedative affects of the anesthetic benzyl alcohol. In this chapter, I demonstrate the absence of a metabolic component to rapid benzyl alcohol tolerance.

## Methods

### Fly stocks

All flies were raised on standard cornmeal/molasses/agar medium. Flies were kept in a room at a constant temperature (20°C) and 12:12 hour light:dark cycle. Flies that emerged from pupae were collected over a period of two days, transferred to fresh food containing bottles, and allowed to age between three to four days. These animals were divided up into groups of ten females each under light $CO_2$ anesthesia, and tested the following day. In this way, all flies are roughly between five to seven days of age.

### Coating vials with benzyl alcohol

Clear glass vials (30 ml) with a spherical bottom were coated with 200 uL of a 0.4% benzyl alcohol in acetone solution and rotated continuously at room temperature for 20 minutes to allow the volatile acetone (vapor pressure at 20 °C is 185.6 mm Hg) solvent to evaporate, leaving an even coating of the non-volatile benzyl alcohol (vapor pressure at 20 °C is .07 mm Hg) behind.

### Tolerance Assay

In the first exposure six groups of ten age matched female flies were incubated in benzyl alcohol coated vials for fifteen minutes, while their control counterparts were exposed to clean benzyl alcohol free vials. Twenty-four hours after this exposure both the experimental and control

groups were incubated in benzyl alcohol coated vials for fifteen minutes. Flies were allowed to recover in clean, benzyl alcohol free, clear glass, vials.

**Behavioral analysis**

Behavioral recovery was quantified from images taken of the animals recovering, at one frame every ten seconds. Flies are normally negatively geotactic; this behavior ceases while they are sedated. Automated image processing software is used to detect when the flies recover from sedation and return to climbing the walls of their vials [71]. Briefly, the software subtracts images of each vial from the image where all flies are sedated. This resulting subtracted image is void of background and only contains white flies which have recovered and begun climbing. The number of non-black pixels are then counted to generate a quantity that represents the number of flies recovered and returned to negative geotaxis. For each vial, the value at each time point is normalized to a value that represents complete recovery, giving a percent recovery curve. The percent recovery of each vial within a population is then averaged as a function of time, and plotted with error bars describing the standard error of the mean (SEM). A left-ward shift in the recovery graph indicates the presence of tolerance to benzyl alcohol. A statistically significant difference between the two curves is determined using logrank analysis.

**Gas Chromatography**

Liquid phase gas chromatography was used to assay for the concentration of benzyl alcohol in flies. The samples were prepared for gas chromatography by transferring flies every 15 min after the cessation of benzyl alcohol exposure to a .2mL gas chromatography vial containing 100uL of acetone, and capped. A standard dilution series of benzyl alcohol in acetone was prepared for the generation of a standard curve. The samples and the benzyl alcohol dilutions were then loaded into the carousel of a SRI 311H 42-vial liquid autosampler, with the vial pressure set to 6 psi and the tray pressure at 60 psi and the injection volume was set to 3uL. The autosampler is connected to an SRI 310 GC through an on column injection, the samples are injected into a 60 meter Restek MXT®-1 column with the hydrogen, carrier (He), and air pressures set to 8,16, 8 psi respectively and detected through a flame ionized detector during the course of each run. The oven temperature was programed in SRI PeakSimple software for Windows and was set to hold at 50°C for 1 minute and then ramps to 200°C at 20°C per minute, and held at 220°C for 5 minutes. The voltage changes in the detector were acquired and analyzed using SRI PeakSimple software. Benzyl alcohol concentration is determined by interpolating benzyl alcohol peak area to a standard curve that relates benzyl alcohol peak area to molarity of solution. To determine the molarity of benzyl alcohol per fly, the molarity of the 100uL fly containing acetone solution is multiplied by 12.5, derived from 100uL total

volume/8 uL $H_2O$ per 10 flies. Non-linear regression and dose response analysis were performed in Prism 5.

## Results

### Benzyl alcohol clearance is independent of previous exposure

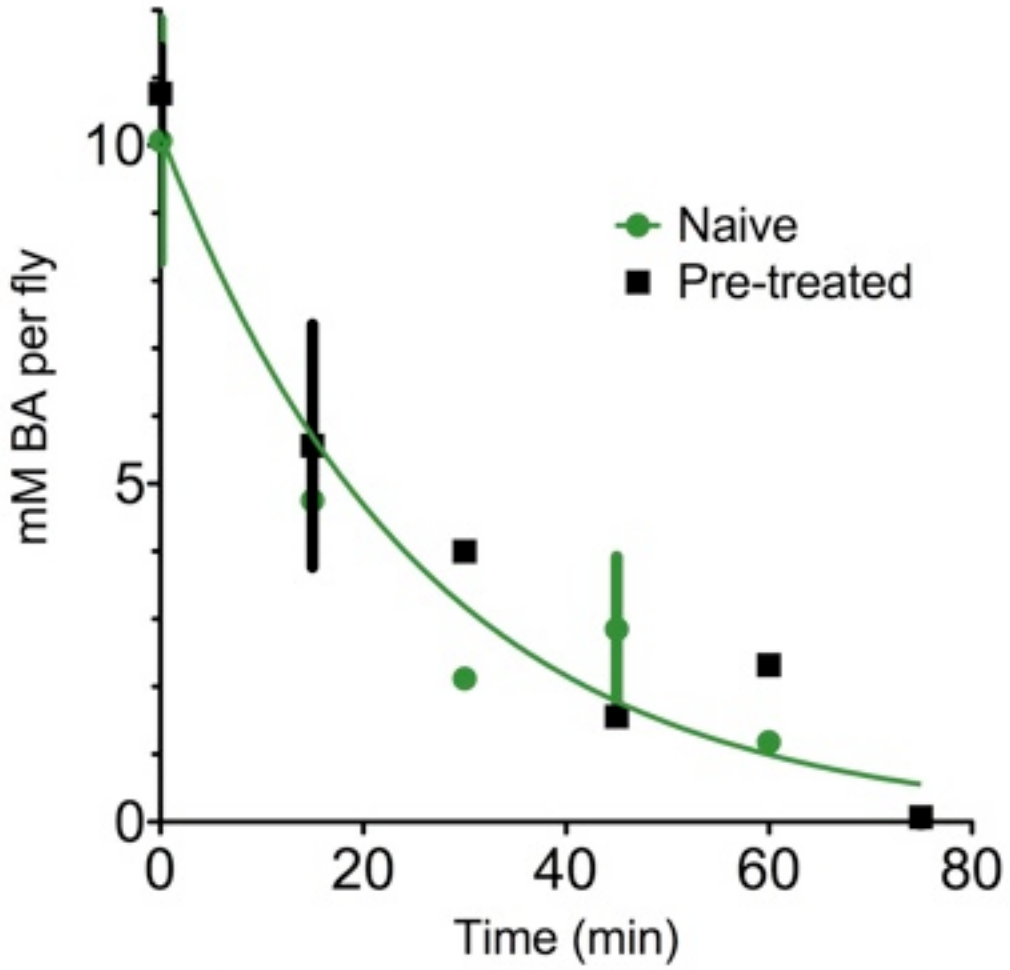
Using gas chromatography, a commonly used analytical technique to quantify solute composition, I measured the clearance of benzyl alcohol from flies during the course of recovery from sedation. It is important to note that the concentrations of benzyl alcohol presented here reflect the concentration of benzyl alcohol within the entire animal. I measured benzyl alcohol concentrations in pre-treated and naive animals immediately before the termination of benzyl alcohol application and every 15 minutes subsequent, until it became undetectable by 75 minutes (Figure 2.1). These measurements were plotted as a function of time after the cessation of benzyl alcohol exposure and fit to a one-phase exponential decay function to produce the following model:

$$[BA]= y_0\, e^{-kt} \text{ or } [BA]= 10\, e^{-.04(t)}$$

Where $y_0$ is the initial concentration of benzyl alcohol of approximately 10mM, k is the rate constant, and t is the time in minutes. Based on the rate constant (k),0.038 $min^{-1}$, the half-life of benzyl alcohol in fruit flies was estimated to be 17.9 minutes:

$$life_{1/2}\ [BA]= \ln(2)/k, \text{ or } .69/.038= 17.9 \text{ minutes}$$

This model fits the decay of benzyl alcohol in both the naive and pre-treated animals (Figure 2.1), indicating that benzyl alcohol clearance is unaffected by prior sedation.

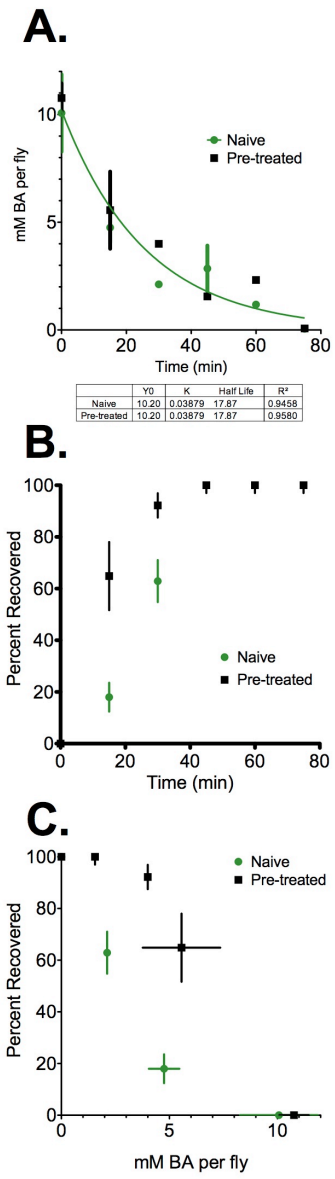



| | Y0 | K | Half Life | $R^2$ |
|---|---|---|---|---|
| Naive | 10.20 | 0.03879 | 17.87 | 0.9458 |
| Pre-treated | 10.20 | 0.03879 | 17.87 | 0.9580 |

Figure 2.1 Benzyl alcohol metabolism is independent of previous exposure. The chart above is of the clearance of benzyl alcohol from animals that have been pre-treated (black) and naive controls (green). Both sets of data can be fit by a single model (green line). The table below the chart, is the output of the parameters that describe the best fit line from the graph above. The $R^2$ values in both cases are > 0.9.

**Right-ward shift in dose-response relationship due to rapid tolerance**

Animals that have been exposed to a prior benzyl alcohol challenge recover more rapidly than their naive counterparts. This type of tolerance is evident by a left-ward shift in the recovery plots where the percentage of flies climbing the sides of the vial is expressed as a function of time (Figure 2.2B). In order to demonstrate that the tolerance under study is in fact a pharmacodynamic form of rapid tolerance and does not have metabolic origins, I expressed behavioral recovery as a function of benzyl alcohol concentration. The expression of behavioral recovery (Figure 2.2B) as a function of benzyl alcohol concentration yields a dose-response curve to the hypnotic effects of benzyl alcohol (Figure 2.2C). When comparing the dose-response curve of pre-treated animals to that of control animals, a right-ward shift indicates that pre-treated animals recover at higher doses of benzyl alcohol, thus rapid tolerance to benzyl alcohol is not a pharmacokinetic mechanism. The negation of a metabolic contribution to tolerance indicates that the rapid tolerance in these studies arises solely from functional behavioral tolerance.

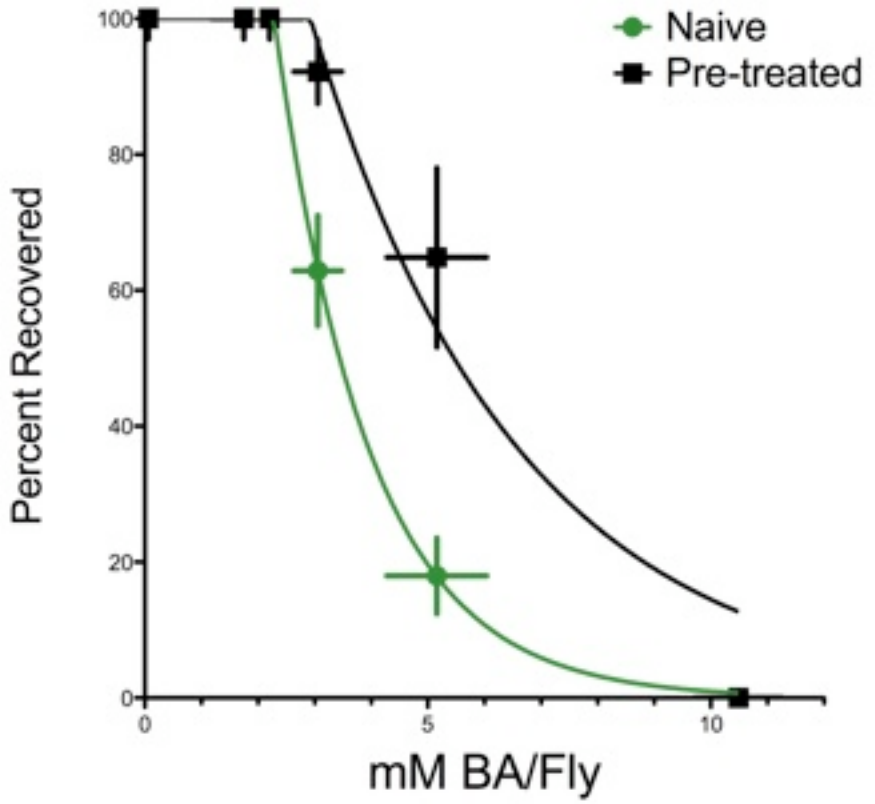



| | Y0 | K | Half Life | $R^2$ |
|---|---|---|---|---|
| Naive | 10.20 | 0.03879 | 17.87 | 0.9458 |
| Pre-treated | 10.20 | 0.03879 | 17.87 | 0.9580 |

Figure 2.2 Pre-treated animals recover at higher doses of benzyl alcohol. A) is the same graph presented in figure 2.1, but is re-presented here for the purposes of comparison. B) is a graph of behavioral recovery from benzyl alcohol sedation, the green points represent the recovery of naive animals while the black points represent the recovery of pre-treated animals. C) is a dose-response curve, behavioral recovery is expressed as a function of benzyl alcohol cencentration. The green points are naive animals and the black points are pre-treated animals. The x and y error bars represent SEM.

**Dose-response curve analysis of rapid tolerance**

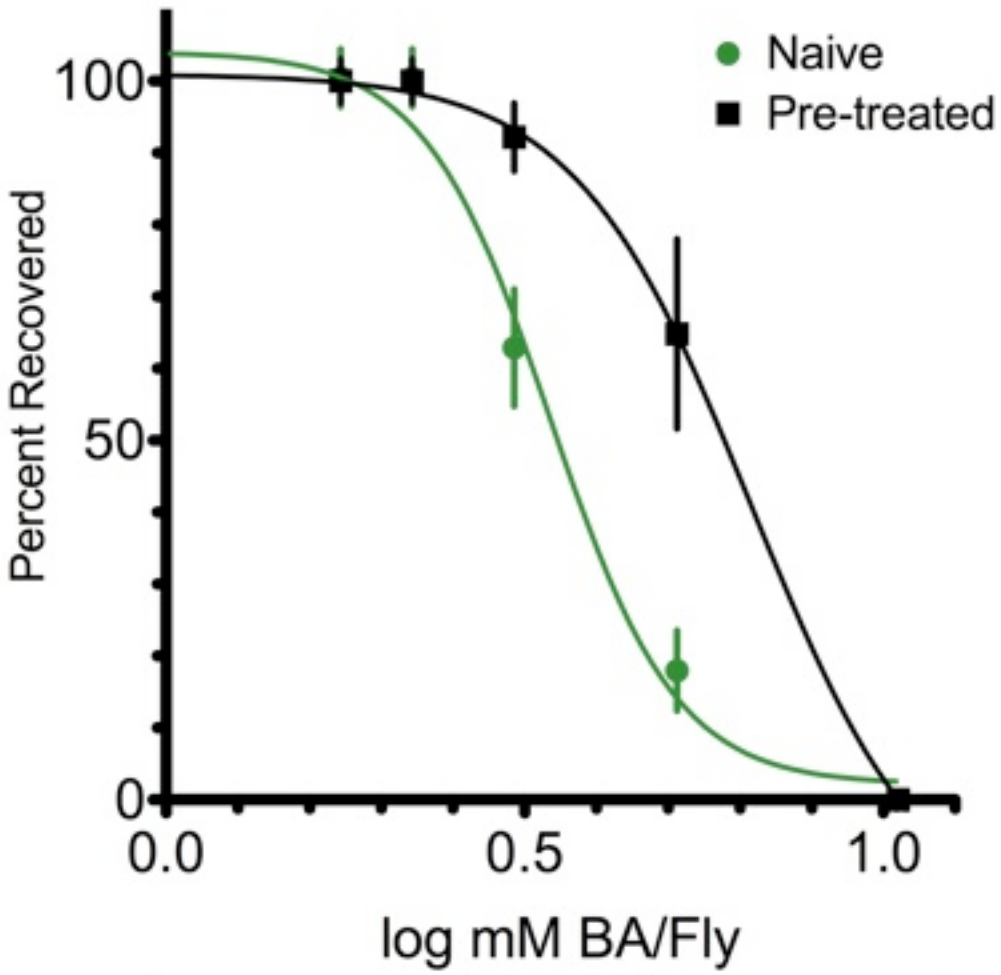
Before presenting the parameters generated from the dose-response curve analysis, I demonstrate that the two dose-response curves discussed here are statistically different. This difference is evident as a large reduction in the $R^2$ value results upon cross-constraining the parameters of one curve on the fitting of the other. The parameters used here are the ones that were generated from modeling each of the dose-response curves using single-phase non-linear regression analysis. These parameters are summarized in the table appended to Figure 2.3. Notice that the $R^2$ value for each curve is larger than 0.9 (table in Figure 2.3, left-most $R^2$ value). After constraining the fitting parameters of the naive dose-response curve to those generated from the pre-treated dose-response curve, the $R^2$ value becomes 0.6354 (middle $R^2$ value) and constraining the pre-treated curve to the parameters of the naive curve results in an $R^2$ value of 0.7442 (right most $R^2$ value). The inability to cross-substitute the models demonstrates that the two dose-response curves are sufficiently different, indicating that pre-treated dose-response curve is right-ward shifted.




| | K | X0 | $R^2$ | $R^2$ | $R^2$ |
|---|---|---|---|---|---|
| Naive | 0.5994 | 2.285 | 0.9999 | 0.9999 | 0.7442 |
| Pre-treated | 0.2729 | 2.924 | 0.9634 | 0.6354 | 0.9634 |

Figure 2.3 Dose-response curves fit using a plateau followed by single phase decay kinetics. The black points are data that represent the dose-response of pre-treated animals, and the black line represents the best fit line. The green points are data that represent the dose-response of naive animals, and the green line represents the best fit line. The bottom table summarizes the parameters of the best fit curve generated by non-linear regression. The three columns labeled $R^2$ are the $R^2$ values generated without cross-constraining (left) and after cross-constraining the parameters of one data set onto the fit of the other (middle and right).

Figure 2.4 is a plot of the log dose-response used to derive the effective dose at which 50% of the flies are recovered ($EC_{50}$). Here I demonstrate that pre-treatment with benzyl alcohol causes an increase in $EC_{50}$, from 3.43 mM in the naive state to 6.553 mM in the tolerance state. This increase in $EC_{50}$ can be expressed as an $EC_{50}$ ratio of pre-treated $EC_{50}$ over the naive $EC_{50,}$ also known as the $EC_{50}$ shift. Here, the $EC_{50}$ shift is 1.9 indicating that the concentration of benzyl alcohol required to sedate 50% of pre-treated animals is almost twice as high as the concentration that sedates 50% of naive animals (Figure 2.4).




| | Naive | Pre-treated |
|---|---|---|
| **HillSlope** | -4.942 | -3.514 |
| **EC50** | 3.430 | 6.553 |

Figure 2.4 Two-fold increase in $EC_{50}$ caused by benzyl alcohol tolerance. The graph is a plot of log dose-response curves generated to calculate $EC_{50}$ values for the recovery of naive animals (green) and pre-treated animals (black). Below is a summary of the $EC_{50}$ and Hill Slope results.

## Discussion

The relationship of benzyl alcohol clearance as a function of time follows first order kinetics. Since the reaction rate of first order reactions is dependent on the concentration of the substrate, benzyl alcohol clearance is dependent on benzyl alcohol concentration. Even though a metabolite of benzyl alcohol has yet to be definitively demonstrated in flies, in a mammalian model McCloskey et. al. (1986) showed that benzyl alcohol is oxidized to benzaldehyde by alcohol dehydrogenase. Aldehyde dehydrogenase then oxidizes benzaldehyde to benzoic acid, and with the addition of glycine, benzoic acid is converted to hippuric acid for secretion in urine [59].

The main findings discussed here are that: tolerant populations receive the same dose of benzyl alcohol as naive ones, thus benzyl alcohol tolerance does not arise from a change in the rate of absorption nor metabolism of benzyl alcohol. I also was able to quantify the half-life of benzyl alcohol in Drosophila to be about 18 minutes. Finally, I demonstrate an estimated two fold shift in $EC_{50}$ after the development of tolerance to benzyl alcohol.

I propose that benzyl alcohol sedation arises through similar mechanisms as the ones described for commonly used clinical anesthetics and alcohols. Benzyl alcohol has been demonstrated to inhibit action potential generation and propagation [80], block the activity of

voltage-activated $Na^+$ channels [28] and ligand gated channels such as the NMDA receptor [81] and acetylcholine receptor [8]. Furthermore, the doses that produce sedation are similar to the clinically relevant concentration of other alcohols and anesthetics such as diethyl ether (10 mM), nitrous oxide (17.4 mM), xenon (3.7 mM), n-butnol (10 mM) and n-pentanol (2.9 mM) [47], and is similar to one described by Staiman, A. and Seeman, P. (1977) [80]. We view tolerance as an adaptive mechanism that allows the nervous system to overcome the inhibitory affects of benzyl alcohol by increasing the excitability of the neuronal regions that render the animal sedated when inactivated.

# Chapter 3: Synaptic function and the development of tolerance

## Introduction

Drug tolerance is defined as a reduction in the effect of a drug caused by prior drug exposure [5,16,77]. There are two physiologically distinct origins of tolerance. Pharmacokinetic tolerance or metabolic tolerance arises from an increase in the rate of drug clearance or reduced drug uptake, while pharmacodynamic or functional tolerance is an adaptive process that resists the effects of the drug. Functional tolerance that alters a neurally-based behavior is of great interest to neurobiologists because it is assumed to be the product of a change in the activity of the nervous system. Functional tolerance is further subdivided into three categories, acute, rapid and chronic [5,10,38]. These categories are distinguished based on the paradigm used to induce and detect them. Acute tolerance arises during exposure to the drug. Rapid tolerance is the tolerance that exists following a single drug exposure and persists after drug clearance while chronic tolerance is produced by multiple or prolonged bouts of drug exposure.

Functional tolerance is an important component in the addictive process. Users who have acquired tolerance require a larger dose of the drug to achieve the same behavioral effect. Unfortunately, for most abused drugs, tolerance does not always occur to undesired toxic side effects of the drug and as a result the increased consumption needed to achieve a

particular behavioral endpoint leads to increased organic damage [63]. Tolerance is also an endophenotype of addiction and as such, an understanding of the the mechanisms underlying tolerance may provide insight into the mechanics of addiction [51].

Tolerance arises to a wide variety of sedating and abused substances. Chemicals that induce unconsciousness vary in their physical and chemical properties, but all drugs that cause sedation directly or indirectly alter signaling in the nervous system. Anesthetics are dirty drugs in that they affect the activity of a wide variety of proteins including components of the synaptic release machinery, neurotransmitter-gated ion channels and voltage-gated ion channels [70]. However, the relative contribution of these targets towards anesthesia and sedation are not yet clear. In general, anesthetics act by reducing excitatory synaptic transmission and enhancing inhibitory synaptic transmission leading to a net reduction in neuronal activity [29]. Functional tolerance is a homeostatic response to a reduction in neural activity that opposes the sedative effects of anesthesia by increasing neuronal excitability. Which can result from the augmentation of excitatory inputs and a decrease in inhibitory ones [88].

Mutant analysis in Drosophila has identified genes important for the normal response to anesthetics and alcohol [18,21,26,62,68,90]. Because of the strong conservation of gene sequence and function between insects and mammals, genes identified in flies typically serve the same role in mammals, and *vice versa*. Thus, mutant analysis in Drosophila is a

promising approach for identifying genes that are important in anesthetic responses in both flies and mammals.

The experiments discussed here focus on the mechanisms that underlie rapid tolerance to the anesthetic benzyl alcohol. Like other anesthetics, benzyl alcohol has been demonstrated to inhibit channel function [8,28,80,81].

In this study I ask if functional benzyl alcohol tolerance is solely a response to reduced neural activity. To answer this question I use temperature-sensitive paralytic mutations to depress neuronal excitability. I then investigate the role of vesicle fusion and fission in the development of tolerance, and finally ask if a neural blockade prevents the acquisition of benzyl alcohol tolerance.

## Methods

### Fly stocks

All the flies were raised on standard cornmeal/molasses/agar medium. Flies were kept in a room at a constant temperature (20°C) and 12:12 hour light:dark cycle. Flies that emerged from pupae were collected over a period of two days, transferred to fresh food containing bottles, and allowed to age between three to four days. Then these animals were divided up into groups of ten females each under light $CO_2$ anesthesia, and tested the following day. All flies are roughly between five to seven days of age at the time of testing. The genotypes of mutant stocks used were *para*$^{ts1}$, *shi*$^{ts1}$, *shi*$^{ts2}$, *comt*$^{st17}$, and *Syx1A* $^{3-69}$ the wild type stock was Canton S.

### Coating vials with benzyl alcohol

Clear glass vials (30 ml) with a spherical bottom were coated with 200 uL of a 0.4% benzyl alcohol in acetone solution and rotated continuously at room temperature for 20 minutes to allow the volatile acetone (vapor pressure at 20 °C is 185.6 mm Hg) solvent to evaporate, leaving an even coating of the non-volatile benzyl alcohol (vapor pressure at 20 °C is .07 mm Hg) behind.

**Tolerance Assay**

In the first exposure six groups of ten age matched female flies were incubated in benzyl alcohol coated vials for fifteen minutes, while their control counterparts were exposed to clean benzyl alcohol free vials. Twenty-four hours after this exposure both the experimental and control groups were incubated in benzyl alcohol coated vials for fifteen minutes. Flies were allowed to recover in clean, benzyl alcohol free, clear glass, vials.

**Behavioral analysis**

Behavioral recovery was quantified from images taken of the animals recovering, at one frame every ten seconds. Flies are normally negatively geotactic; this behavior ceases while they are sedated. Automated image processing software is used to detect when the flies recover from sedation and return to climbing the walls of their vials [71]. Briefly, the software subtracts images of each vial from the image where all flies are sedated. This resulting subtracted image is devoid of background and contains only white images of flies which have recovered and have begun climbing. The number of non-black pixels are then counted to generate a quantity that represents the number of flies recovered and returned to negative geotaxis. For each vial, the value at each time point is normalized to a value that represents complete recovery, giving a percent recovery curve. The percent recovery of each vial within a population is then averaged as a function of time, and plotted with error bars describing the standard error

of the mean (SEM). A left-ward shift in the recovery graph indicates resistance to the sedative affects of benzyl alcohol. A statistically significant difference between the two curves is determined using logrank analysis.

**Manipulation of temperature sensitive mutations**

The temperature sensitive paralytics, *para*$^{ts1}$, *shi*$^{ts1}$, and *shi*$^{ts2}$ were incubated at the nonpermissive temperature of 30°C for five hours. A cotton plug containing 3 mL of water was inserted into vial to prevent the animals from desiccating during the incubation at elevated temperature. The flies were then placed into food vials and allowed to recover and recuperate overnight until they received a sedating dose of benzyl alcohol the following day. The heat-shock of *para*$^{ts1}$, after benzyl alcohol sedation received the same heat-shock protocol as described above immediately after removal from the benzyl alcohol containing vials, while *comt*$^{st17}$ were incubated at 37°C for 1hr immediately following sedation.

**Electrophysiology**

Adult, four to seven day old female flies were fixed using super glue (Loctite$^{TM}$) with their heads and 1/4 of their thorax protruding out of a pipette tip, a pulled capillary tube was used to glue the thorax to the pipette tip and the head to the thorax. The pipette tip was then fixed to a magnetic chemistry clamp, and the fly oriented such that the head is in the

horizontal position. Two 75 um (FHC Inc, Bowdoinham, ME) diameter insulated tungsten wire electrodes were implanted into the brain and eye of the fly using micro-manipulators (Narishige, Tokio, Japan). The positive recording electrode was placed in the brain while the negative and ground electrode were placed in the eye. The recordings were then amplified by a 10X headstage and further amplified by a Microelectrode Amplifier Model 1800 (A-M systems, Inc., Carlsborg, WA), and digitized using National Instruments® cRIO 9215 and acquired on a Dell® inspiron 1150 laptop computer using a Labview program written by Sari Andoni (unpublished). Drug delivery was achieved by the application of small amount of benzyl alcohol through a pulled capillary on to the abdomen of the fixed animal.

**Feeding N-ethylmaleimide**

Clear glass vials containing an NEM or 1% sucrose vehicle soaked filter paper were prepared by the application of 300uL of the appropriate solution to the filter paper in a manner that causes the filter paper to adhere to the sides of the vial. Adult four to seven day wild type females were then transferred to the drug or vehicle containing glass vials. The animals were allowed to feed for twenty four hours prior to testing.

**Gas Chromatography**

Liquid phase gas chromatography was used to assay for the concentration of benzyl alcohol in flies. The samples were prepared for gas chromatography by transferring flies to a .2mL gas chromatography vial containing 100uL of acetone, and capped. A standard dilution series of benzyl alcohol in acetone was prepared for the generation of a standard curve. The samples and the benzyl alcohol dilutions were then loaded into the carousel of a SRI 311H 42-vial liquid autosampler, with the vial pressure set to 6 psi and the tray pressure at 60 psi and the injection volume set to 3uL. The autosampler is connected to an SRI 310 GC® through an on column injection, the samples are injected into a 60 meter Restek MXT®-1 column with the hydrogen, carrier (He), and air pressures set to 8,16, 8 psi respectively and detected through a flame ionized detector during the course of each run. The oven temperature was programed in SRI PeakSimple software for Windows and was set to hold at 50°C for 1 minute and then ramps to 220°C at 20°C per minute, and held at 220°C for 5 minutes. The voltage changes in the detector were acquired and analyzed using SRI PeakSimple software. Benzyl alcohol concentration determined by interpolating benzyl alcohol peak area to a standard curve that relates benzyl alcohol peak area to molarity of solution. To determine the molarity of benzyl alcohol per fly, the molarity of the 100uL fly containing acetone solution multiplied by 12.5, derived from 100uL total volume/8 uL $H_2O$ per 10 flies.

## Results

Benzyl alcohol is an anesthetic that is well-tolerated by flies. Like other anesthetics, benzyl alcohol suppresses neural activity in a reversible manner. Figure 3.1A shows local field potentials recorded from the fly brain prior to benzyl alcohol application, and Figure 3.1B is ten minutes after the application of a small amount of benzyl alcohol to the thorax. This application produces approximately a 20 minute reduction in neuronal signaling that is followed by a return to the pre-anesthetic state as the animal recovers from sedation.

Prior exposure to this drug induces long-lasting functional tolerance that can be scored in behavioral assays [26,92]. An example of functional tolerance is presented in Figure 3.1C. These curves represent the recovery of two populations of animals from benzyl alcohol sedation. One population is recovering from their first sedation with benzyl alcohol (green) while the other population (black) is recovering from their second consecutive sedation. Notice that recovery from the second sedation is more rapid than recovery from the first sedation. This leftward shift in the sedation recovery curves is a behavioral manifestation of tolerance to benzyl alcohol sedation.

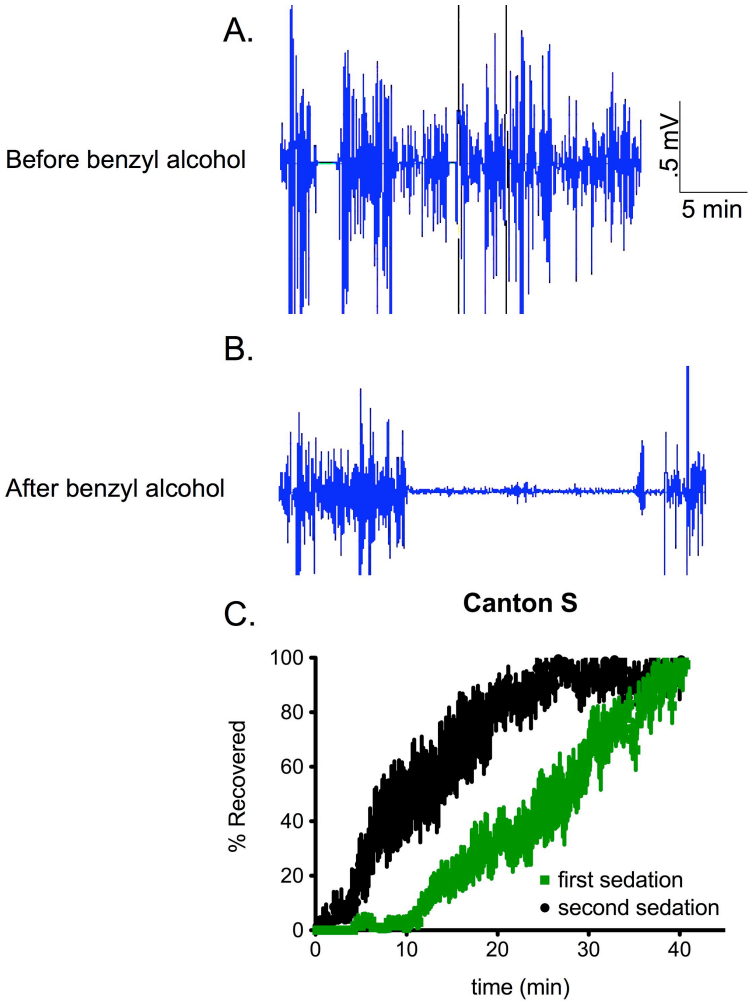



Figure 3.1 Effects of benzyl alcohol anesthesia. A) Local field potentials recorded within the fly brain prior to benzyl alcohol application. The scale indicates dimensions of both A and B. B) Benzyl alcohol anesthesia depresses the activity of the nervous system. Shown are local field potentials recorded within the fly brain as the animal is sedated with benzyl alcohol. The reduction in bursting activity indicates that benzyl alcohol suppresses neuronal signaling. C) A tolerance assay showing a behavioral effect of benzyl alcohol sedation. Two populations of animals are sedated with benzyl alcohol and moved to a fresh-air environment at t=0 min. Shown are recovery curves for a population of animals recovering from their first sedation with benzyl alcohol (green) and a population recovering from their second sedation (black). Plotted are the percentage of flies climbing the walls of the vial. Flies recovering from their second sedation recover more rapidly than do animals recovering from their first sedation. Error bars are standard error of the mean. For these behavioral assays, significant differences between curves was determined by log-rank test if $p < 0.05$

**Reduction in neural signaling does not phenocopy tolerance**

The observation that cross tolerance to benzyl alcohol is induced by exposure to other sedative agents with distinct chemical moieties such as chloroform and ethanol, led us to ask if tolerance was merely a response to reduced neural activity. To test whether reduced neural signaling is sufficient to phenocopy tolerance we suppressed neural activity through the use of temperature sensitive paralytic mutations, and tested them the following day for benzyl alcohol resistance.

We inhibited neuronal signaling at the level of action potential generation and propagation using a mutation in the major Drosophila $Na^+$ channel, *paralytic*. This mutation, *para*$^{ts1}$, reduces the voltage-activated $Na^+$ channel current [66]. The *para*$^{ts1}$ allele is a temperature sensitive mutation, at the restrictive temperature of 30°C neuronal action potential generation is inhibited resulting in paralysis. The blockade is completely reversible and animals recover rapidly and function normally once returned to the permissive temperature (20°C) [24].

We also tested neuronal blockades induced at the level of the synapse using mutant alleles of the *shibire* and *comatose* genes. The *shibire* gene encodes a Drosophila GTPase dynamin, which is required for vesicle recycling. Shibire acts as a “pinchase” that forms a ring around the neck of clathrin coated pits, and upon hydrolysis of GTP mediates vesicle fission. When viewed under electron microscopy after incubation at the restrictive temperature, synapses of *shi*$^{ts1}$ exhibit clathrin coated pits with

exaggerated necks and a reduced number of vesicles [46]. These studies demonstrated that the paralytic phenotype of temperature sensitive mutations in the *shibire* locus is caused by a reduction in neurotransmitter containing vesicles due to the inhibition of local vesicle recycling at the synapse.

Comatose is the Drosophila neuronal specific NEM Sensitive Factor (NSF) [27], whose main function is in disassembly of the SNARE complex after fusion or priming of vesicles. *comt*$^{st17}$ is a hypomorph mutation that results in paralysis at 37°C and is coincident with the accumulation of assembled SNARE complex [86], leading to an activity dependent reduction in neurotransmitter release [41].

With these mutants we temporarily reduced neuronal signaling and asked whether the reduction induces resistance to benzyl alcohol sedation, thereby phenocopying benzyl alcohol tolerance. Such a result is expected only if tolerance to benzyl alcohol sedation is a response to a gross reduction in neural activity. Temperature-sensitive mutants were incubated at 30°C for five hours with the exception of *comt*$^{st17}$ (see methods) and then returned to the permissive temperature and allowed to recover. Twenty four hours later, the benzyl alcohol sensitivity of the heat-treated animals was compared to the benzyl alcohol sensitivity of age- and sex-matched animals that had been maintained at the permissive temperature. Figure 3.2 shows that a reduction in neuronal signaling did not induce a tolerance-like phenotype in any of the mutant lines.

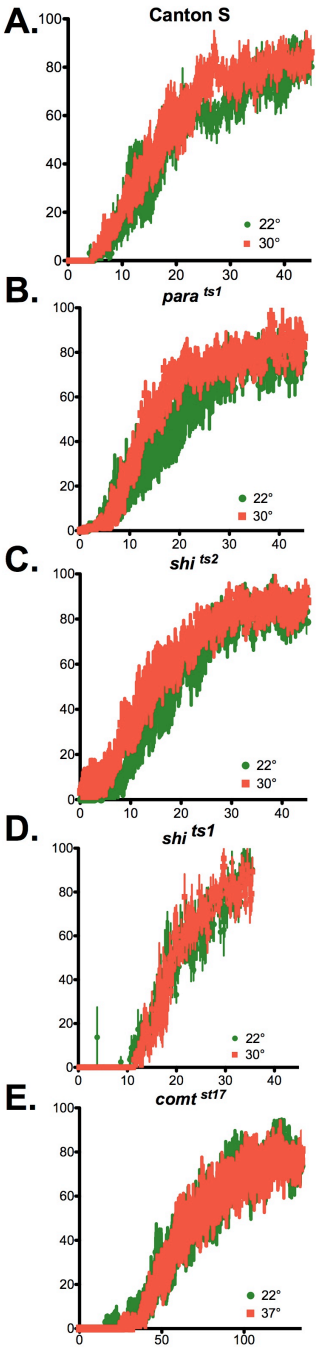



Figure 3.2 Reduction in neural activity does not phenocopy tolerance. A-E are recovery plots of Canton S controls (A) and four temperature sensitive mutations *para*$^{ts1}$ (B) *shi*$^{ts1}$ (C) *shi*$^{ts2}$ (D) and *comt*$^{st17}$ (E), the day after incubation at elevated their restrictive temperatures (red), compared to their control counterparts that were maintained at room temperature (green).

**shibire mutations interfere with the development of tolerance**

We tested the *paralytic* and *shibire* mutants for their ability to acquire tolerance at the permissive temperature. These mutant animals are considered to have nominally normal behavior at the permissive temperature [42,44,53,78,89]. While all mutants are predicted to reduce or eliminate neural activity at elevated temperatures we found that they have different effects on the capacity to acquire tolerance to benzyl alcohol sedation at the permissive temperature.

Although, mutation-induced paralysis did not phenocopy tolerance, we observed that some of these mutations interfered with the normal acquisition of benzyl alcohol tolerance. We found that the *para*$^{ts1}$ mutation did not compromise the capacity to acquire tolerance (Figure 3.3B), while mutations that affect vesicle recycling *shi*$^{ts1}$ and *shi*$^{ts2}$ (Figure 3.3D and Figure 3.3E) did interfere with the acquisition of tolerance. These finding led us to conclude that proper vesicular endocytosis is required for the development of tolerance.

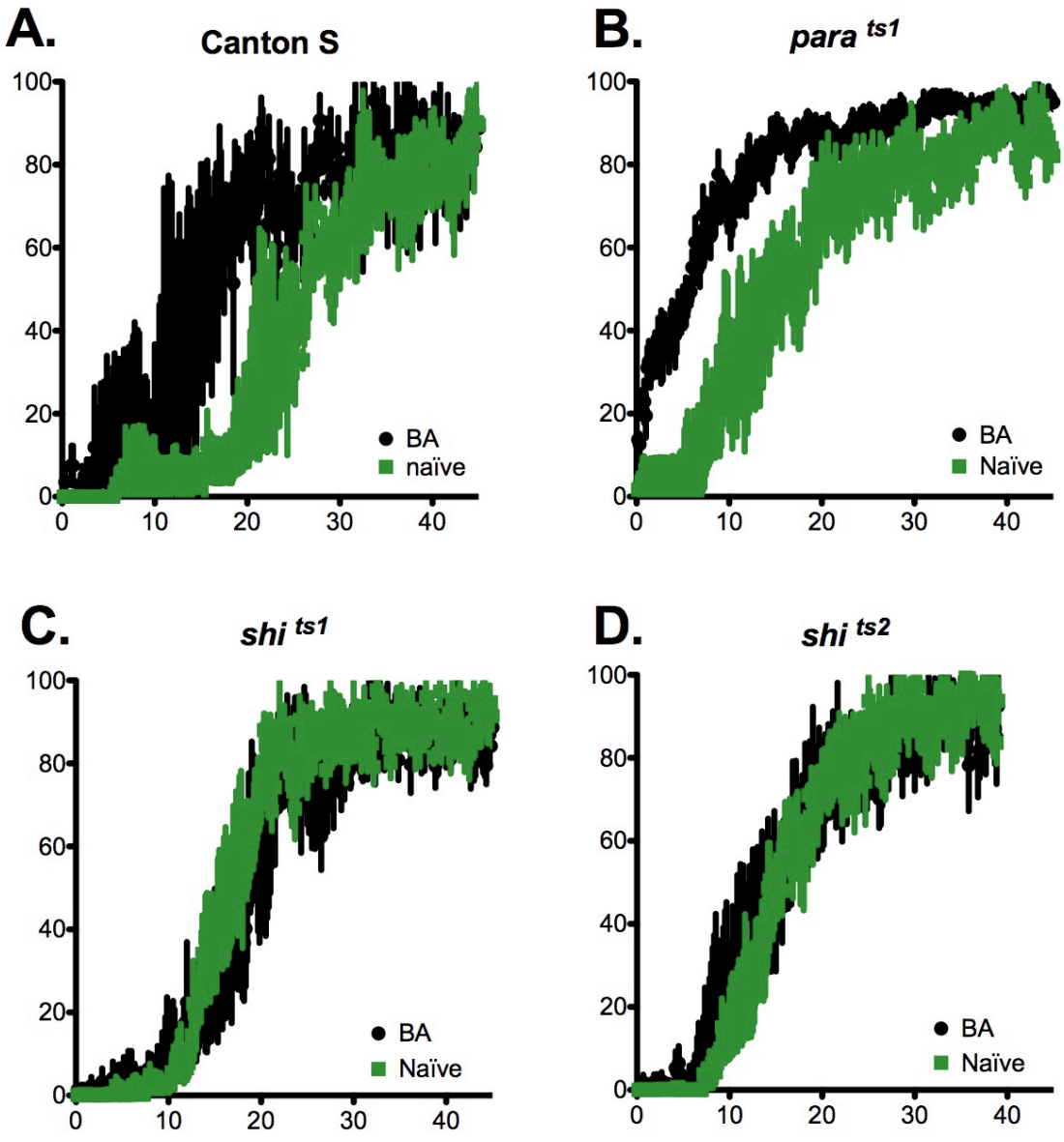



Figure 3.3 shibire mutants fail to develop tolerance. A-D are recovery plots of Canton S controls (A) and three temperature sensitive mutations *para* $^{ts1}$ (B), *shi*$^{ts1}$(C), *shi*$^{ts2}$(D) after benzyl alcohol sedation (black), compared to their naive counterparts that were not sedated (green).

**N-ethylmaleimide interferes with the development of tolerance**

The demonstration that mutations that perturb synaptic function also prevent the development of tolerance, led us to examine the affects of pharmacological manipulation of the synapse on benzyl alcohol tolerance. For this set of experiments adult wild type flies were fed sugar water laced with the sulfhydryl alkylating agent, *N*-ethylmaleimide (NEM). NEM is a widely used reagent that inhibits various synaptic processes including vesicle recycling and fusion [13,56,57].

Before delving into the effects of NEM on tolerance it is important to establish the concentration of NEM that causes the minimal effect on basal behavior. This dosage was established by feeding animals three concentrations of NEM-laced food, 1, 0.1, and 0.01 mM NEM, while taking images over the course of hours as they consumed NEM (Figure 3.4). The animals fed 1mM NEM demonstrated a marked reduction in geotactic behavior four hours after their transfer to NEM containing food, and did not return to normal climbing over the course of 24 hours. Similar to the 1mM NEM fed animals the 0.1 mM NEM caused a reduction in climbing, although to a lesser degree, and appeared healthy after twenty four hours, while the 0.01 mM NEM dose did not cause any noticeable change in behavior. Based on these results I proceeded to test the the effects of 0.1 and 0.01 mM NEM on benzyl alcohol tolerance and resistance.

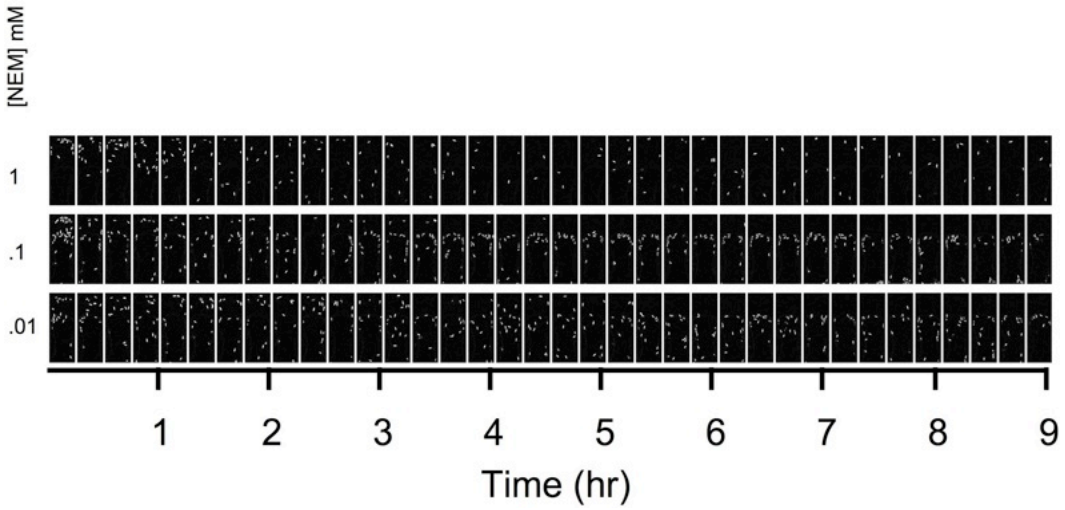



Figure 3.4 High doses of NEM impair fly health and climbing. Animals were fed three different doses of NEM 1mM (top row), 0.1mM middle row and .01mM bottom row. Images were taken and subtracted to determine climbing behavior over the course of NEM feeding. The white pixels represent flies able to climb.

I wished to determine whether inhibiting vesicle recycling and fusion by feeding NEM would affect sensitivity to a first exposure to benzyl alcohol. Therefore I fed flies two doses of NEM, and tested them 24 hours later for benzyl alcohol sensitivity. Flies fed 0.01 mM NEM displayed no difference in sensitivity to benzyl alcohol compared to control flies. While flies fed 0.10 mM NEM displayed resistance (Figure 3.5A and B). To test the effects of NEM on tolerance, I used the 0.01 mM dose as it did not cause a change in initial sensitivity.

I set up three treatment groups of flies, where the feeding of NEM was varied with respect to the first benzyl alcohol sedation: the first group was fed NEM before and after their first benzyl alcohol sedation (Figure 3.5C), the second group was fed NEM before their first benzyl alcohol sedation (Figure 3.5D), and the third group was fed NEM after their first benzyl alcohol sedation (Figure 3.5E). I then examined how long it took each of these groups of flies to recover from a second exposure to benzyl alcohol, compared to flies that were fed NEM but not exposed to the first sedating dose of benzyl alcohol. Flies fed NEM after their first benzyl alcohol sedation but not before, acquired tolerance just like control flies, while flies fed NEM before their first benzyl alcohol sedation failed to acquire tolerance. A very seductive explanation for these data is that a NEM-sensitive process performs an essential step in the production of tolerance and that this process occurs during but not after the first benzyl alcohol

sedation. Once this stage of been completed NEM thereby looses it capacity to interfere with the acquisition of tolerance.

This interpretation is predicated on the assumption that the dose of NEM consumed before and after benzyl alcohol sedation is the same. However, the possibility certainly exists that benzyl alcohol sedation can reduce or interrupt feeding behavior. To test if benzyl alcohol sedation alters feeding behavior I conducted a defecation assay for food consumed after sedation. This was accomplished by transferring benzyl alcohol sedated animals to vials containing food mixed with a food coloring tracer. Thus the number of colored defecations is an indication of food consumed after sedation. After comparing the number of food coloring containing fecal matter droplets left overnight on the sides of the vials between treated flies and untreated controls no obvious difference was observed (Figure 3.5F), Furthermore, the latency to the initial defecation remained the same (data not shown).

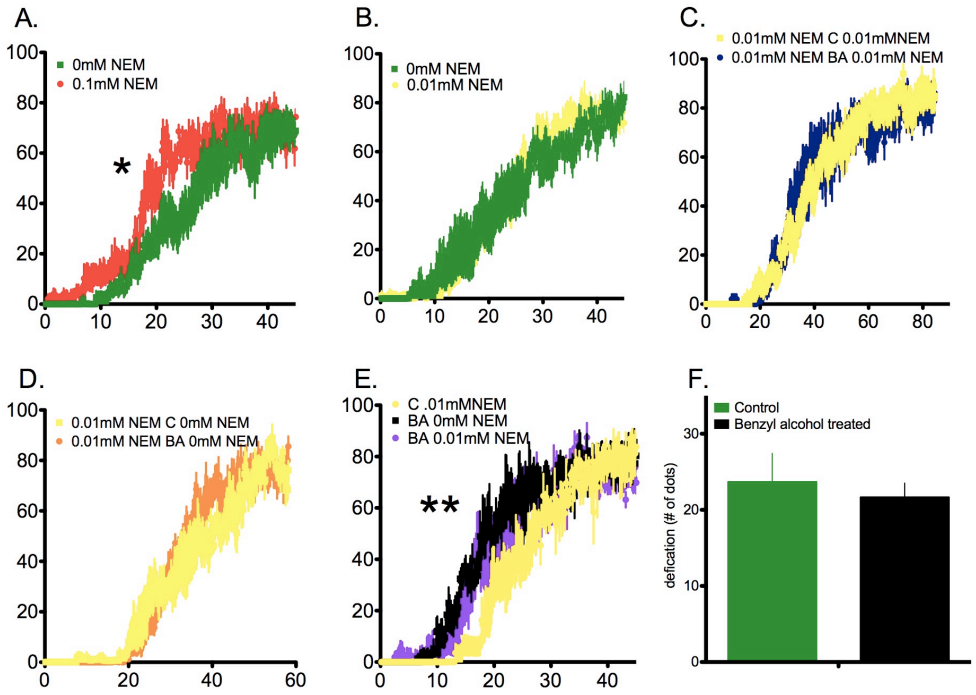



Figure 3.5 Feeding NEM prevents the acquisition of tolerance. A) 0.1 mM NEM food causes resistance. Flies that have consumed 0.1 mM NEM (red) recover more rapidly from benzyl alcohol sedation than animals not fed NEM (green). B) Feeding 0.01mM NEM does not cause a change in sensitivity to benzyl alcohol. Flies that have consumed 0.01 mM NEM (yellow) recover from benzyl alcohol sedation at the same time as animals not fed NEM (green). C) Feeding 0.01 mM NEM before and after the initial benzyl alcohol sedation prevents tolerance. Flies that have consumed 0.01 mM before and after the initial benzyl alcohol sedation (blue) recover at the same time as naive animals fed 0.01 mM NEM (yellow). D) Feeding 0.01 mM NEM before the initial benzyl alcohol sedation prevents tolerance. Flies that have consumed 0.01 mM before the initial benzyl alcohol sedation (orange) recover at the same time as naive animals fed 0.01 mM NEM (yellow). E) Feeding 0.01 mM NEM after the initial benzyl alcohol sedation does not prevent tolerance. Flies that have consumed 0.01 mM after the initial benzyl alcohol sedation (orange) recover at the same time as benzyl alcohol tolerance animals (black) and more rapidly that naive animals fed .01 mM NEM (yellow). F) Benzyl alcohol sedation does not alter feeding behavior. Defecation assay for food consumed after benzyl alcohol sedation (black) does not from control (green).

**A mutation in syntaxin interferes with tolerance**

The inhibition of tolerance by a drug that interferes with vesicular fusion led us to suspect that mutations that perturb vesicle fusion should also interfere with tolerance. Here I test two mutations known to interfere with vesicular fusion *Syx1A* $^{3\text{-}69}$ and *comt* $^{st17}$.

The *syntaxin*1A gene, is a Drosophila Soluble NSF Associated Protein Receptor (SNARE) protein whose role in synaptic transmission is required in the target membrane for vesicular fusion [53]. Unlike the other temperature sensitive paralytics tested in this study, *Syx1A*$^{3\text{-}69}$ is a gain of function mutation that enhances the elevated frequency of spontaneous mini-excitatory junctional potentials (mini-ejps) [49]. Also unlike the other temperature sensitive mutants tested this mutant displayed “bottom dwelling” behavior and were paralyzed but not motionless at 37°C.

Here we demonstrate that a mutation affecting *syntaxin*1A, *Syx1A*$^{3\text{-}69}$, prevents the manifestation of tolerance due to prior benzyl alcohol sedation (Figure 3.6B), while a mutation that affects the Drosophila NSF, *comt*$^{st17}$, develops tolerance due to prior exposure (Figure 3.6A).

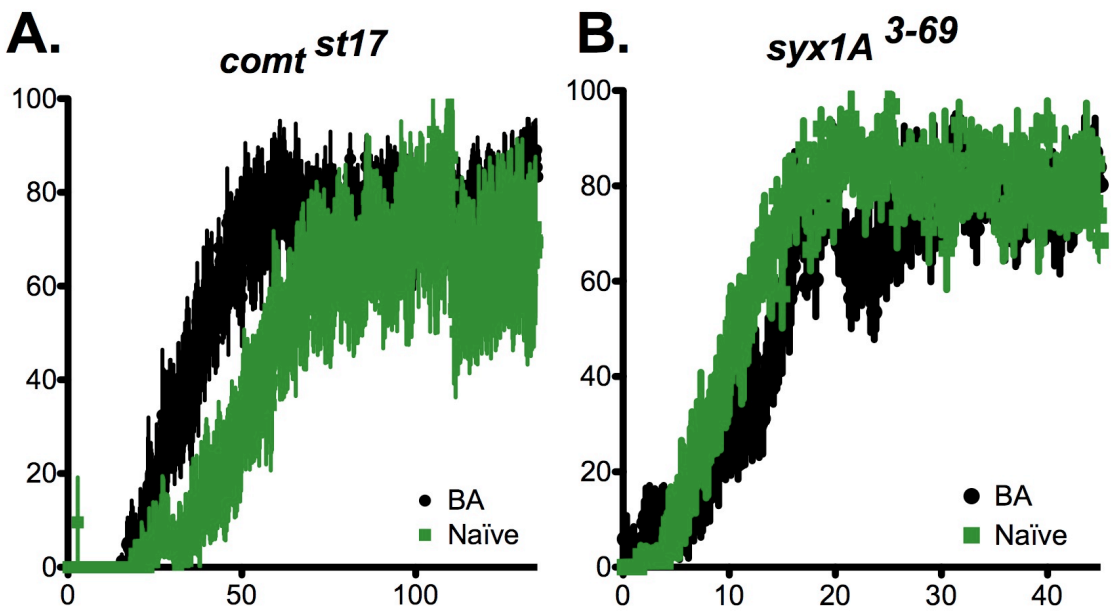



Figure 3.6 syx1A$^{3-69}$ mutants fail to acquire tolerance to benzyl alcohol. A) comt$^{st17}$ mutants develop tolerance to benzyl alcohol. B) syx1A$^{3-69}$ do not acquire tolerance to benzyl alcohol.

**Benzyl alcohol tolerance is cell autonomous after sedation:**

Emergent properties arise from interactions of neurons in a network, and the interactions of networks within the brain to culminate in the production of a task that a single neuron or network cannot achieve alone. Neuronal and network outputs rely on action potential propagation and chemical transduction to integrate various inputs to generate a novel output. When neuronal plasticity involves cell to cell communication it is also considered to be an emergent property. In a simplified model of neuronal plasticity, a change in neuronal firing frequency can alter ion channel composition and function in the post-synaptic cell altering the electrical properties of the target neuron. If the development of tolerance requires neuronal communication, the signal that induces the development of tolerance has cellular origins, and should be dependent on neuronal signaling.

Here I examine the role of neuronal signaling after benzyl alcohol sedation in the development of tolerance. To do so, I used two temperature sensitive mutant lines that can acquire tolerance, *para*$^{ts1}$ and *comt*$^{st17}$, to inhibit action potential generation and vesicular fusion after sedation with benzyl alcohol. These flies were kept at a permissive temperature, then exposed to their first dose of benzyl alcohol for 15 minutes, and immediately placed at the restrictive temperature for the recovery period. If inhibition of signaling between neurons prevents the acquisition and manifestation of tolerance, the purple curves in Figure 3.7

would be right-ward shifted compared to the tolerant population (black curves). However, inhibition of cell signaling after benzyl alcohol sedation did not prevent the acquisition of tolerance (Figure 3.7A and B) thus indicating that neuronal substrates that mediate this behavioral plasticity do so in a cell autonomous manner, and not an emergent one. Furthermore, we could eliminate residual benzyl alcohol as the cause of tolerance as it is completely metabolized during the prolonged period of temperature mediated inhibition of signaling (Figure 3.7C).

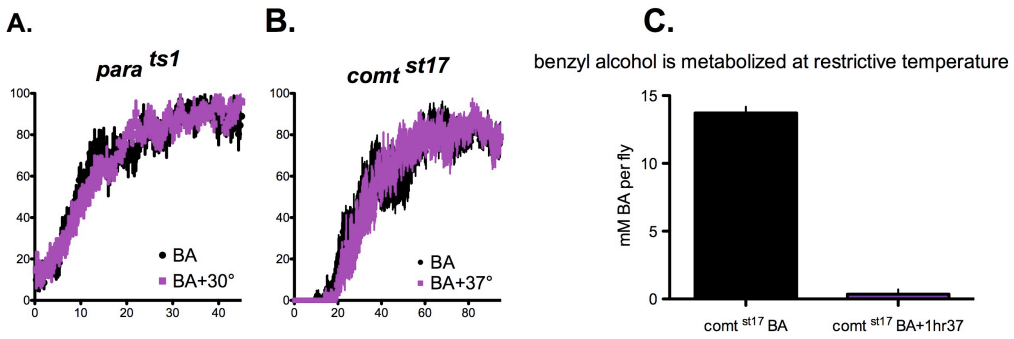



Figure 3.7 Inhibition of neuronal signaling after benzyl alcohol sedation does not prevent tolerance. A) Inhibition of action potential generation after benzyl alcohol sedation does not prevent tolerance. *para*$^{ts1}$ incubated at 30°C for five hours immediately following benzyl alcohol sedation (purple) recover at the same time as a tolerant population (black). B) Inhibition of vesicle fusion after benzyl alcohol sedation does not prevent tolerance. *comt*$^{st17}$ incubated at 37°C for one hour after their initial benzyl alcohol sedation (purple) recover at the same time as a tolerant population (black). C) Risidual benzyl alcohol is not responsible for tolerance after inhibition of signaling. Benzyl alcohol in *comt*$^{st17}$ mutants is completely metabolized during the one hour incubation at 37°C (purple) from initial levels (black).

**Discussion:**

Previously, we showed that the capacity to acquire tolerance to sedation with benzyl alcohol requires the neuronal expression of the $Ca^{2+}$-activated $K^+$ channel encoded by the *slowpoke* gene. Furthermore we found that *slowpoke* mRNA is induced after benzyl alcohol sedation, and that artificial induction of *slowpoke* from a transgene was sufficient to produce resistance to the drug [26]. These results suggest that tolerance is a homeostatic response to reduced neuronal activity. Here I demonstrate that a drug-free reduction in neural activity does not evoke the tolerance phenotype, that the neuronal basis of tolerance involves proper synaptic function and that tolerance is a cell-autonomous response to drug exposure.

The finding that we cannot reproduce a state of resistance by simply inhibiting neuronal signaling indicates that the homeostatic mechanism that produces tolerance to benzyl alcohol is not triggered by a mere reduction in neural excitability. There are at least two possible explanations as to why paralysis evoked using temperature sensitive mutants did not cause resistance to benzyl alcohol: The first possibility is that the gross temperature-induced inhibition of signaling does not contain the same structure as the pattern of neuronal activity caused by benzyl alcohol sedation. Thus a difference between the electrical characteristics of benzyl alcohol sedation, and mutation-induced inhibition prevents

temperature-induced inhibition from activating the cellular mechanisms that produce tolerance. The second possibility is that tolerance requires the specificity of the drug interaction with its targets, which in turn initiates a cascade leading to an increase in neuronal activity and tolerance.

The discrepancy between the electrical signatures produced by benzyl alcohol sedation and mutant induced inhibition of neuronal signaling provides an unlikely explanation for the inability of reduced signaling to phenocopy tolerance. According to such a hypothesis feeding 0.01mM NEM, and mutations in *shibire* and *syntaxin*1A should alter the pattern of electrical activity caused benzyl alcohol sedation as to prevent tolerance. While mutations in *paralytic* and *comatose* retain all the necessary electrical constituents of benzyl alcohol sedation to develop tolerance, furthermore 0.1mM NEM fed animals are resistant to benzyl alcohol in the absence of sedation. It is more likely that our inability to reproduce drug tolerance through the use of temperature sensitive paralytic mutations indicates that the interaction of benzyl alcohol with specific targets is required for the initiation of tolerance. Such a relationship is reminiscent of a drug and receptor interaction where a signal that originates at the site of the receptor bound to the drug leads to the activation of cellular components responsible for increasing neural activity and the subsequent development of tolerance.

According to the drug-receptor model cross-tolerance between drugs should occur if the drugs affect overlapping sites, or induce the same

biochemical signal to induce a similar adaptive mechanism. In the case of anesthetics and drugs that depress neuronal signaling, this adaptive mechanism should result in an increase in neuronal signaling, reducing the sedative effects of a different anesthetic.

Our mutant and pharmacological analysis of tolerance point to the synapse as an important site for the development of tolerance. This result is congruent with the plethora of studies that identify synaptic processes in adaptive mechanisms including neuronal homeostasis [20]. Presently our working hypothesis is that tolerance to agents that blunt neuronal signaling, such as anesthetics, is caused by an adaptive mechanism that strengthens neuronal signaling within the neural substrates relevant for the end points required for recovery from sedation and the return to normal geotaxis.

Upon testing the effects of interference with the vesicle cycle on tolerance I found that exposure to NEM and mutations in the *shibire* and *syntaxin*1A loci, prevent the acquisition of tolerance. The mutations tested here are temperature sensitive paralytic mutations, that are seldom associated with a phenotype at permissive temperatures [32,49,69,76]. The demonstration that these mutations are defective in the ability to acquire tolerance at permissive temperatures suggests that the mutated function is required to a larger degree during the development of tolerance than during normal conditions. The outlier in this set of experiments was a comatose mutation, *comt*$^{st17}$, which did acquire tolerance. This puzzling

observation may be due to the fact that the *comt*$^{st17}$ allele is a hypomorph that paralyzes at higher temperature (37°C) than the *paralytic* and *shibire* mutants. This mutation may not interfere with dNSF function in vesicle fusion in the same manner as NEM exposure. Furthermore, the site affected by this mutation, the first of two ATPase domains found in NSF1, may not be required for the development of tolerance. Another possibility that could explain the discrepancy between the key players involved in exocytosis, *comatose* and *syntaxin*1A, is that *comatose* is not directly involved in exocytosis rather it functions in recycling the SNARE complex and priming vesicles for exocytosis, as oppose to *syntaxin*1A which directly mediates vesicle fusion with the plasma membrane.

The contributions of endocytosis and exocytosis to anesthetic tolerance remain unclear. These mechanisms have been demonstrated to alter the composition of ligand and voltage-activated ion channels found in the membrane, a process crucial to many forms of neural adaptations [23,36,43]. One may expect that vesicle recycling and fusion would be most relevant to the expression of tolerance, or to one of the final steps in the development of tolerance. However, based on the findings that feeding NEM and inhibition of vesicle fusion in *comt*$^{st17}$ following the initial benzyl alcohol sedation do not prevent the development of tolerance suggests that vesicle fusion and fission act during the initial inductive phase of tolerance. It is worth noting that *syntaxin*1A, a component of the vesicle-release pathway, has been identified as a target of the commonly used

general anesthetic isoflurane and is identified here as a tolerance mutant [31]. Future experiments devoted to dissecting the steps leading to anesthetic tolerance may provide insight into their specific roles in this process.

The final observation discussed here is that inhibition of neuronal signaling for a prolonged period of time after the first drug exposure does not prevent the manifestation of tolerance. This demonstrates that tolerance is a cell autonomous mechanism, where the cells that mediate this behavioral plasticity can adapt to benzyl alcohol sedation independent of neuronal signaling. Because tolerance is cell autonomous the transcriptional and electrophysiological phenotypes associated with the development of benzyl alcohol tolerance should be recapitulated *in vitro* or *in situ* settings. This study would entail the use of neuronal cell culture or whole brain explants, which can be perfused with a benzyl alcohol containing solution. Epigenetic and transcriptional analysis of the nuclear responses *in vitro* to benzyl alcohol exposure should mirror the changes observed *in vivo*. Furthermore, Drosophila cell culture and explants are well suited for electrophysiological recordings, I would expect an increase in spontaneous or evoked firing rate after benzyl alcohol exposure. This would provide a method to study the intricacies of tolerance on a finer scale that cannot be achieved *in vivo*.

# Chapter 4: Post-translation modification of slowpoke mediates rapid tolerance

## Introduction

Anesthetics are known to reduce neuronal function by inhibiting excitatory inputs and activating inhibitory ones. The ability to develop tolerance to anesthetics is based on a homeostatic mechanism that increases the excitability of relevant neuronal substrates in the presence of the anesthetic. Generally, the excitability of a neuron is determined by its release of neurotransmitter. This emergent function is under heavy regulation from the types and number of ion channels and ion channel modulators expressed, to the intricate control of the vesicle-release and recycling machinery. The $Ca^{2+}$-activated $K^{+}$ channel, slowpoke, has been demonstrated to play a critical role in the development of tolerance to benzyl alcohol. Slowpoke is a BK type ion channels that integrates some of the basic components of neuronal signaling $Ca^{2+}$, metabolites, and changes in membrane potential. This channel is also considered to have a large conductance of 100-250 pS, 10 times that of voltage-activated potassium channels. These diverse and potent properties put this molecule at the center of our studies relating to the homeostatic development of tolerance.

In Drosophila, *slowpoke* expression is under the transcriptional regulation of at least 5 distinct promoters that regulate expression in the nervous system, muscle, midgut, and trachea, in combination with

alternative splicing a fly can express 1512 unique isoforms, if heterotetramerization is permitted then the possibilities of *slowpoke* holochannels expressed in an animal become incomprehensible [6]. Finally the *slowpoke* channel also undergoes direct post-translational modifications, which regulate the channel's voltage and $Ca^{2+}$ sensitivities and kinetics.

Previously we identified that neuronal expression of the $Ca^{2+}$-activated $K^{+}$ channel gene, *slowpoke*, as necessary for the development of tolerance to benzyl alcohol, and that tolerance was coincident with the upregulation of a neuronal isoform of *slowpoke*. This conclusion however, does not preclude a transcription-independent role of the *slowpoke* protein in the development of tolerance. In the endogenous state it is difficult to pinpoint the relevant mechanisms that mediate *slowpoke* dependent tolerance due to the complexity described above.

To simplify the state of *slowpoke* expression I test a transgenic line of flies that carry a single *slowpoke* cDNA under the regulation of a heatshock promoter in a $slo^4$ mutant background, known as B52H [6], for the ability to develop tolerance. The $slo^4$ mutation is a null mutation that globally prevents the expression of functional channels. In this line the only source of slowpoke mRNA is from the *slowpoke* cDNA containing B52H transgene, thus a single isoform is produced, under the regulation a single constitutively active yet inducible heat shock promoter. Inducing this

transgene causes resistance to benzyl alcohol, indicating that the cDNA expressed by this line is relevant in anesthetic responses.

The $Ca2^{+}$-activated $K^{+}$ channel has been demonstrated to be the target of phosphorylation by CaMKII [54], Akt [15], PKA [95], PI3K [52] Src and Lck [40]. The plethora of kinases that act on the *slowpoke* channel provide further insight into the complexity of *slowpoke* regulation. Here we will explore the ability of the B52H transgene to rescue the inability of $slo^{4}$ mutants to acquire tolerance.

## Methods

### Fly stocks

All flies were raised on standard cornmeal/molasses/agar medium. Flies were kept in a room at a constant temperature (20°C) and 12:12 hour light:dark cycle. Flies that emerged from pupae were collected over a period of two days, transferred to fresh food containing bottles, and allowed to age between three to four days. Then these animals were divided up into groups of ten females each under light $CO_2$ anesthesia, and tested the following day. In this way, all flies are roughly between five to seven days of age. The genotypes of mutant stocks used were B52H a transgenic strain that carries a slowpoke cDNA under the regulation of a heat shock promoter in a *slo*$^4$ background.

### Coating vials with benzyl alcohol

Clear glass vials (30 ml) with a spherical bottom were coated with 200 uL of a 0.4% benzyl alcohol in acetone solution and rotated continuously at room temperature for 20 minutes to allow the volatile acetone (vapor pressure at 20 °C is 185.6 mm Hg) solvent to evaporate, leaving an even coating of the non-volatile benzyl alcohol (vapor pressure at 20 °C is .07 mm Hg) behind.

**Tolerance and resistance assays**

In the first exposure six groups of ten age matched female flies were incubated in benzyl alcohol coated vials for fifteen minutes, while their control counterparts were exposed to clean benzyl alcohol free vials. Twenty-four hours after this exposure both the experimental and control groups were incubated in benzyl alcohol coated vials for fifteen minutes. Flies were allowed to recover in clean, benzyl alcohol free, clear glass, vials. To test for resistance to benzyl alcohol, animals undergone induction of *slowpoke* are compared to uninduced animals, twenty-four hours after induction.

**Behavioral analysis**

Behavioral recovery was quantified from images taken of the animals recovering, at one frame every ten seconds. Flies are normally negatively geotactic; this behavior ceases while they are sedated. Automated image processing software is used to detect when the flies recover from sedation and return to climbing the walls of their vials [71]. Briefly, the software subtracts images of each vial from the image where all flies are sedated. This resulting subtracted image is void of background and only contains white flies which have recovered and begun climbing. The number of non-black pixels are then counted to generate a quantity that represents the number of flies recovered and returned to negative geotaxis. For each vial, the value at each time point is normalized to a value that represents

complete recovery, giving a percent recovery curve. The percent recovery of each vial within a population is then averaged as a function of time, and plotted with error bars describing the standard error of the mean (SEM). A left-ward shift in the recovery graph indicates resistance to the sedative affects of benzyl alcohol. A statistically significant difference between the two curves is determined using logrank analysis.

**Graded induction of slo**

Animals homozygous for a transgene that contains a *slowpoke* cDNA under the regulation of heat inducible promoter and a slowpoke mutant background (B52H) were heatshocked at 37°C and in humidified conditions for variable periods of time.

**RNA isolation**

Total RNA was extracted from each group of 20 to 25 flies six hours after the start of the induction protocol using a single-step RNA isolation from cultured cells or tissue protocol (Ausubel, 1994) with some modifications. Flies were ground in liquid nitrogen into a fine powder and transferred to a 1ml dounce homogenizer containing 1ml of denaturing solution (4M guanidinium thiocyanate, 25mM sodiumcitrate, 0.1M 2-mercaptoethanol, 0.5% sarkosyl) and homogenized slowly for 2 minutes. 0.5ml of the homogenate was transferred into a 1.5ml microfugetube. 50 ul of 2M sodium acetate, pH 4, 0.5 ml of water-saturated phenol/ chloroform 5:1 pH 4.5 (Ambion Inc. Austin, TX) plus 0.1 ml of a 49:1 chloroform: isoamyl alcohol mixture (each from Fisher Scientific, Fairlawn,

NJ) were added, mixed and incubated for 15 min on ice. The suspension was then centrifuged for 15 minutes at 4°C in a microcentrifuge at maximum speed. The upper aqueous phase containing the RNA was transferred to a fresh 1.5 ml microfuge tube. The RNA was precipitated by adding 1 volume of 100% isopropanol, followed by incubation at –20°C for 20 minutes and centrifugation for 10 minutes at 10,000xg. The dried pellet was then washed in 0.5 ml 75% ethanol, vortexed, and incubated for 10 to 15 minutes at room temperature to extract residual guanidinium thiocyanate. The pellet was centrifuged for 5 min at 10,000xg and the supernatant discarded. The pellet was air-dried for 5-10 minutes and resuspended in 0.2 ml DNase buffer, 3 ul of Superase-In RNase inhibitor and 2 ul of RNase free DNase I (Ambion Inc. Austin, TX) and incubated for 30 minutes at 37°C. Subsequently, the sample was extracted with phenol/ chloroform as before followed by an ethanol precipitation. The pellet was then resuspended in 0.1 ml of DEPC – H 2 O and stored at -80°C. RNA quality was determined by electrophoresis in a 1% agarose gel and quantified 260/280 absorption using a nanodrop.

**Quantification of *slowpoke* induction by real time PCR**

The abundance of *slowpoke* expression after induction was determined by quantifying abundance of a *slowpoke* exon C2 relative to a message that reflects the total amount of RNA, *Cyclophilin1* using real-time RT-PCR assay. First-strand cDNA was synthesized from 50 ng of

total RNA, primed with 200 nM of gene specific lower primers for C2 and *Cyclophilin1*, using Superscript II reverse transcriptase (Life Technologies). Each reaction was performed in triplicate from independent RNA samples. Six additional reactions were performed from a dilution series of RNA concentrations (0ng, 25ng, 50ng, 100ng, 200ng and 400ng) produced from the control RNA sample to create a standard curve. The standard curve is used for quantification purposes and to prove the linearity of the assay. The first strand synthesis was then diluted to 1/5th the original concentration and one ul was added to 0.5X Power SYBR® Green PCR Master Mix containing the relevant primers. The primers used to quantify Cyp1 expression were (ACCAACCACAACGGCACTG) and (TGCTTCAGCTCGAAGTTCTCATC). The primers used to quantify C2 expression were (GCTATTTATAATAGACGGGCCAAGT) and (GGAAATCCGAAAGATACGAATGAT). These reactions were conducted in ABI Prism®7700 Sequence Detection System (AppliedBiosystems) thermocycler set to 2 minutes at 95°C followed by 50 cycles of a 30 second denaturing step at 95°C, a 30 second annealing step at 60°C and a 30 second extension step at 72°C. Data was collected at every cycle during the annealing step. Each PCR was performed in triplicate and averaged. mRNA abundance was extrapolated using the standard curve method. Significance was calculated using the Student's t-Test.

## Results

### Induction of a *slowpoke* cDNA causes resistance

To demonstrate that the transgene expressed in B52H causes resistance to the anesthetic benzyl alcohol, I induced *slowpoke* expression to varying degrees based on the duration of the incubation at 37°C (Figure 4.1A), I compared two levels of induction that represent a low level of induction (30 minutes) and a high level of induction (2 hours) to uninduced controls, Figure 4.1 B and C respectively.

Upon testing these different induction protocols I found that the prolonged induction (Figure 4.1C) produced pronounced resistance as compared to the mild induction of *slowpoke* (Figure 4.1B). As wild type non-transgenic animals that underwent the same heat shock protocol were indistinguishable from their controls that were maintained at room temperature (Figure 4.1D and E), the induced resistance caused by the induction of *slowpoke* was not an artifact of the heat shock protocol.

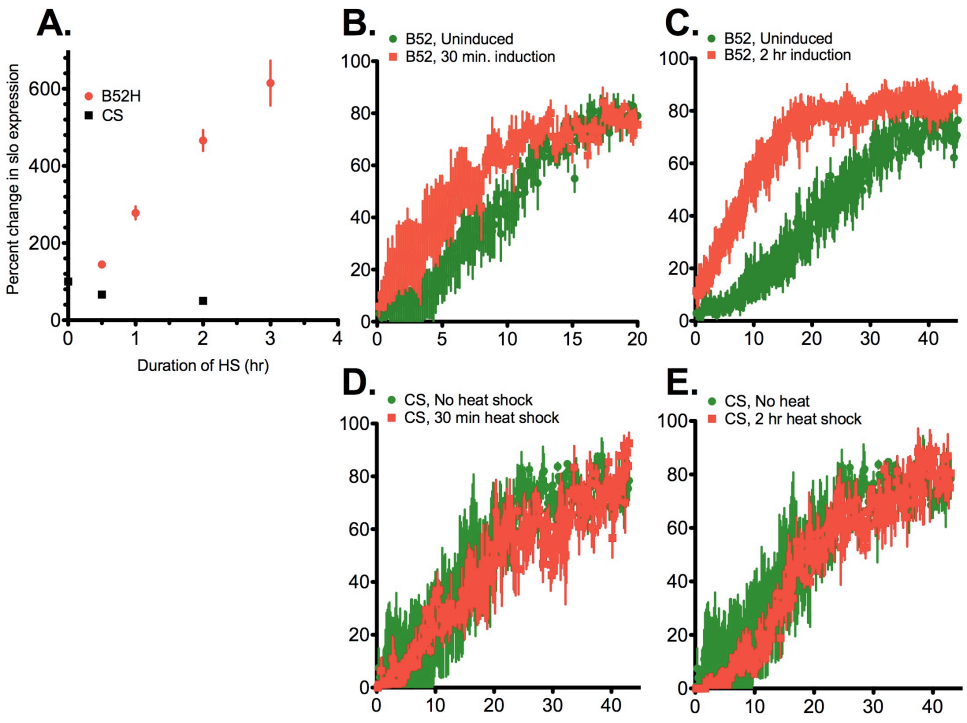



Figure 4.1 Induction of *slowpoke* mRNA causes resistance to benzyl alcohol. A )is a measure of *slowpoke* expression in B52H transgenic flies (red) as a function of incubation time at 37°C, the black points represent *slowpoke* expression in wild type animals after incubation at 37°C. (B and C) are recovery curves of B52H flies induced for 30 minutes and two hours respectively (red) compared to uninduced controls (green). (D and E) are recovery curves of wild type flies that underwent the same incubation protocol as B and C, compared to animals maintained at room temperature (green).

**B52H can acquire tolerance to benzyl alcohol sedation**

Here I test B52H for their ability to acquire tolerance due to pre-treatment with benzyl alcohol. I used this transgenic line to separate the role of *slowpoke* transcription, splicing and heteromultimerization from post translational modifications that may occur after benzyl alcohol sedation. This experiment followed the format of a typical tolerance assay, comparing pre-treated animals to their naive counterparts. The results of this experiment demonstrate that B52H is able to restore the inability of $slo^4$ mutants to develop tolerance (Figure 4.2), thus indicating that the slowpoke channel undergoes post translational modification during the development of tolerance.

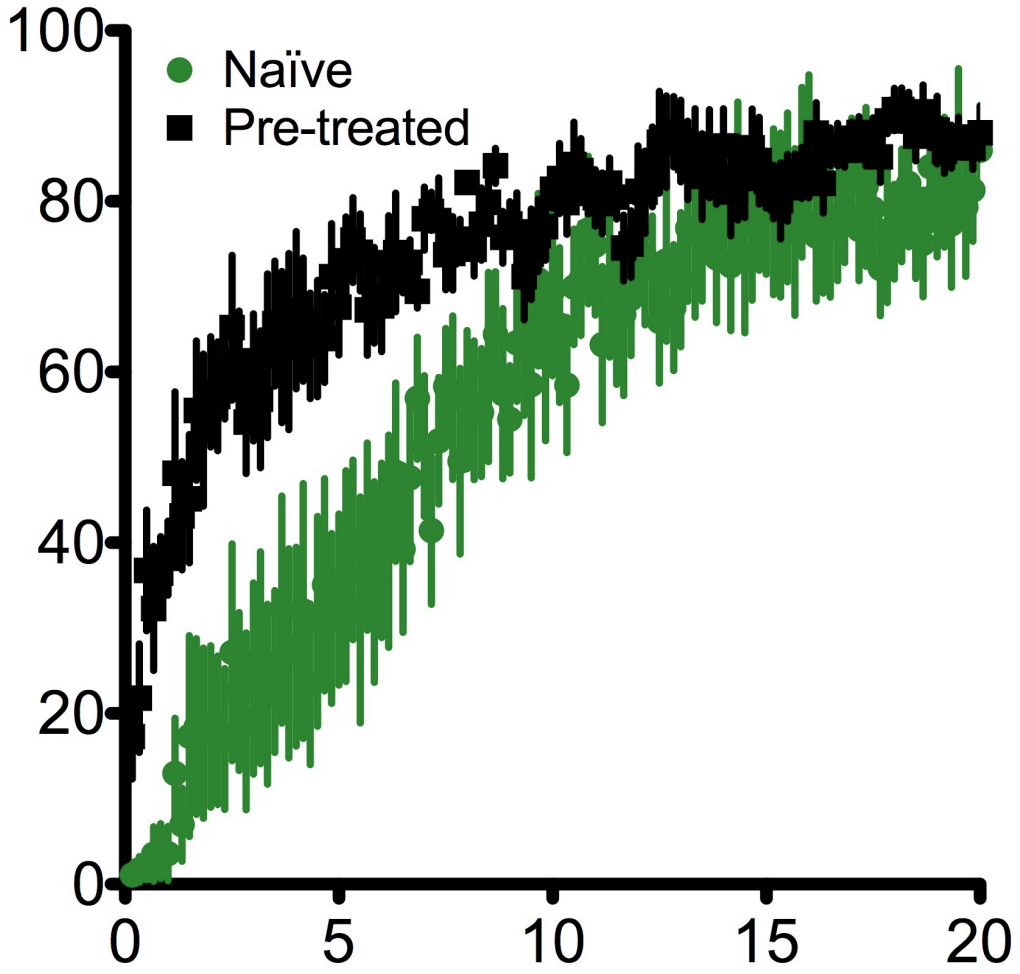



Figure 4.2 B52H can develop rapid tolerance to benzyl alcohol. This graph represents the recovery of B52H flies pre-treated with benzyl alcohol from their second sedation (black) and the recovery naive B52H recovering from their initial sedation (green).

## Discussion

The ability to develop tolerance to benzyl alcohol reflects the ability of the animal to respond to benzyl alcohol sedation by initiating mechanisms that render it more resistant upon subsequent exposures. We have published data indicating that mutations that specifically interfere with slowpoke function prevent the development of tolerance [19,26]. In the past we have focused on transcriptional changes in search of the mechanisms that underlie tolerance [91]. The experiments discussed in this chapter demonstrate that the presence of slowpoke, independent of endogenous transcriptional and splicing regulation, is sufficient to restore the capacity of *slowpoke* mutants to acquire tolerance. This data is consistent with findings from other labs that have identified ethanol dependent changes in slowpoke phosphorylation which act to potentiate channel function [34]. We can conclude that slowpoke channels expressed by B52H contain at least the minimal features required for the development of tolerance. Future experiments that mutate various candidate phosphorylation domains found in the B52H cDNA could prove useful teasing out the post translational components of rapid tolerance. Another possible mechanism that could explain the tolerance rescue of *slo*$^4$ mutants by the constitutive expression of *slowpoke* cDNA is the activation of a micro-RNA system that can alter *slowpoke* translation levels while maintaining a steady level of mRNA.

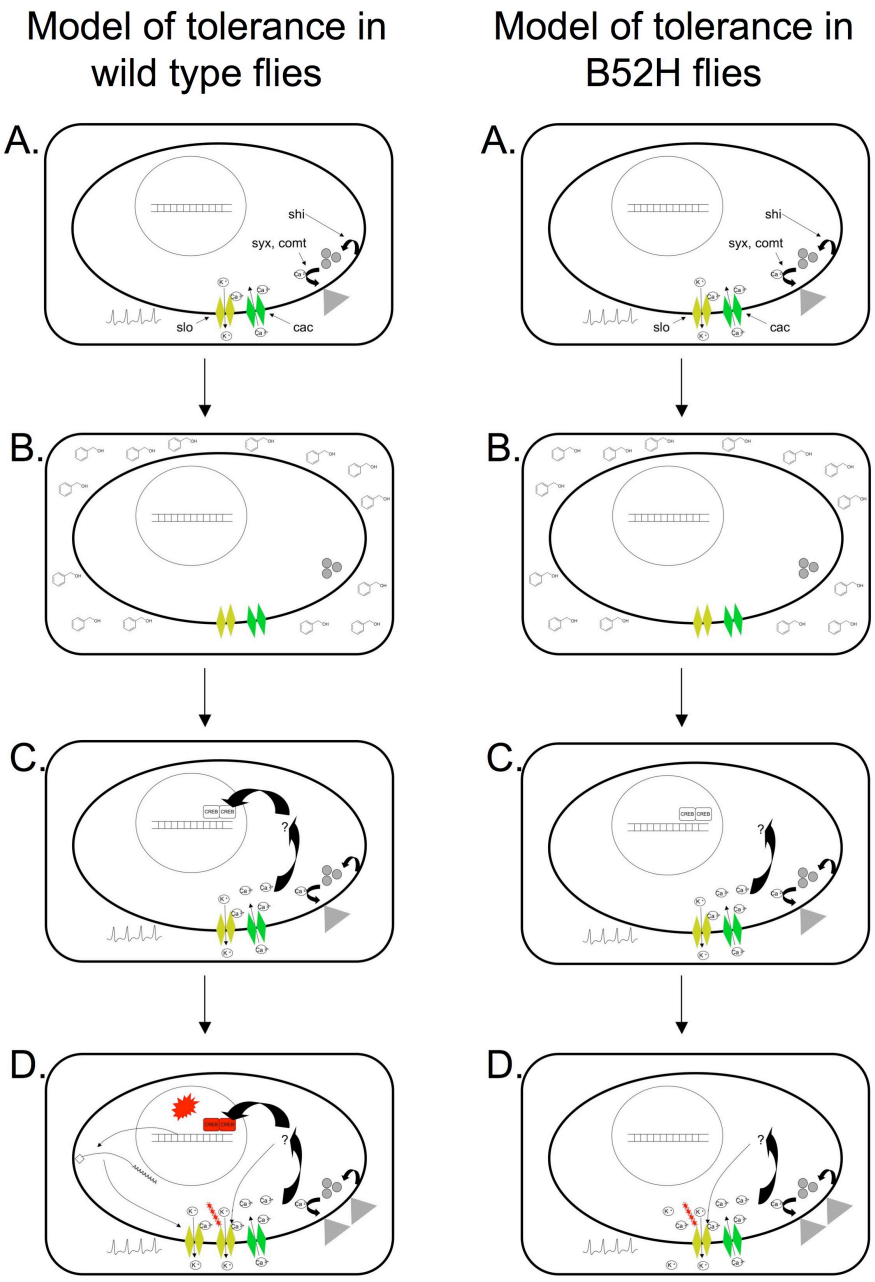



Figure 4.3 Model for the development of tolerance. This figure depicts our model of benzyl alcohol tolerance. The process is separated into four parts (A,B,C, and D), the left column represents tolerance in a wild type nervous system, and the right column represents tolerance in B52H flies. A represents the baseline state in naive animals, B represents the sedated state due to benzyl alcohol inhibition of signaling, C represents the initiation phase that includes that activation of signaling pathways that activate transcription of the *slowpoke* promoter (wild type) and post translational modification of the slowpoke protein (wild type and B52H)

I have provided an illustration of our model of tolerance in both wild type and B52H animals. Figure 4.3 provides the two models, the left side is my model of tolerance in wild type nervous system, while the right half is the tolerance developed by B52H animals. This model breaks up tolerance into four stages. The first state A, represents the baseline activity, B represents the sedated state due to benzyl alcohol inhibition of neuronal signaling, C is the state of initiation of pathways that lead to the potentiated state D, where *slowpoke* expression is upregulated due to epigenetic modification caused by the transcription factor cAMP responsive element binding protein (CREB), and the activation of enzymes that mediate the post translational modifications of slowpoke channels. The main distinction between B52H animals and wild type is found in C, where activation of *slowpoke* transcription is not included in B52H model.

# Chapter 5: Localized induction of *slowpoke* identifies neuronal substrates of benzyl alcohol sensitivity

## Introduction

The studies discussed in the previous chapter demonstrate that global expression of a *slowpoke* cDNA can rescue the loss of tolerance phenotype of *slo*$^4$ mutants, and that global induction of this cDNA causes resistance to benzyl alcohol sedation. The enhancement of the $Ca^{2+}$-activated $K^+$ channel function causes increased neural excitability as seen by the augmentation of repetitive firing (Ghezzi et al. unpublished data) and by reducing seizure threshold in a mammalian system [96]. An increase in *slowpoke* activity leads to an increase in neural excitability, which causes resistance to sedation. Because anesthetics do not affect all brain regions equally, it is unlikely that the entire nervous system adapts uniformly to anesthetic sedation to produce tolerance, thus we should be able to more finely map the relevant regions where *slowpoke* induction causes resistance.

The versatile tools available through the efforts of the fly community have provided a plethora of reagents that allow for the dissection of neuronal circuitry that underlie complex behavioral phenotypes. One such system is the Gal4/UAS system. This binary system, developed by Brand and Perrimon [14] is composed of two parts: the driver and the target. The driver is a yeast transcriptional activator, galactosidase 4 (Gal4), under the control of a temporally and spatially regulated fly gene promoter. The

target is a transgene that responds to Gal4, and is typically the gene whose expression one is interested in regulating. This cloned target is constructed so that it is positioned downstream of a series of Gal4 binding sites, the upstream activating sequence (UAS).

Through the collective endeavors of the Drosophila community, thousands of Gal4 lines are readily available for interested investigators to use. Our lab has constructed a transgenic line that carries a *slowpoke* cDNA under the regulation of UAS. A common application of the Gal4/UAS system involves mating driver lines with a target line to generate offspring heterozygous for both the driver and the target. The offspring of this cross will contain the two components of the Gal4/UAS system and thus the responder will be activated by the presence of Gal4. In the experiments discussed here, I use the Gal4/UAS system to control the expression of a *slowpoke* cDNA in various neuronal structures.

Using the Gal4/UAS system not only allows for spatio-temporal regulation of gene expression, but levels of transcript expression can also be manipulated by the temperature-dependent interaction between the Gal4 transcription factor and its DNA binding partner UAS. Gal4 binds more tightly to the UAS at higher temeratures that at lower ones [35]. Thus allowing for an increase in transcription initiation at elevated temperatures. Overall, this system is versatile and provides a method to control expression levels within a subset of cells.

To identify the neural substrates responsible for anesthetic-dependent behaviors, I targeted the expression of a *slowpoke* cDNA to eleven types of neurons and structures. I expressed *slowpoke* in cells based on the neurotransmitter they synthesize: cholinergic, serotonergic and dopaminergic neurons. I also targeted *slowpoke* expression to seven neuronal structures the: antennal lobes, fan shaped body, dorsal and ventral lateral neurons, ellipsoid body, mushroom bodies, optic lobes and pars intercerebralis. In this chapter I will show that of all the structures tested the only ones to produce a change in anesthetic recovery were the mushroom bodies, the ellipsoid body, and ventral lateral neurons ($LN_V$s).

The mushroom bodies receive inputs from several sensory modalities, including visual, olfactory, and somatosensory. In Drosophila and other insects the mushroom bodies have been found to function in complex behaviors such as place memory [61], associative memory [75], context dependent sensory filtering [55], motor control [60] and the regulation of sleep [69]. This structure is thought to be the insect homologue to the mammalian forebrain [48].

Another structure found to alter anesthetic sensitivity is the central complex. This structure is composed of the ellipsoid body, the superior arch, fan shaped body, and the protocerebral bridge. The ellipsoid body of the central complex has been implicated in the formation of visual and spatial memories [64,67], and the control of locomotor behavior and flight

control [33]. The ellipsoid body is also one of the targets of the Drosophila clock neurons.

Interestingly, the Drosophila clock neurons, $LN_V$s play a role in recovery from benzyl alcohol sedation. The $LN_V$s regulate circadian rhythmcity by coupling autonomously oscillating cells. They release the neuropeptide, pigment dispersing factor (pdf), which serves to regulate the autonomous clocks to generate a single coherent biological rhythm throughout the entire animal [65].

The neural substrates discussed here (the mushroom bodies and ellipsoid body and the $LN_V$s) regulate some of the higher order processes of the insect brain, and are implicated in mediating arousal, a relevant behavior in recovery from anesthesia. Their role in benzyl alcohol resistance and sensitivity will be discussed in this chapter.

## Methods

### Regional Induction of *slowpoke*

Flies homozygous for the UAS-*slowpoke* construct and a mutation that removes *slowpoke* expression, *slo*$^4$, are crossed to animals homozygous to various Gal4 drivers. The offspring of this cross are heterozygous for both UAS-*slowpoke* and the Gal4 driver, and were collected and sorted into groups of 10 animals per vial under light $CO_2$ anesthesia as to include females that were no more than 3 days of age, and allowed to age for another 2 days at 18°C. At which point half of these animals were transferred to 30°C for 3 days, this population will serve as the induced group. After this induction period the resistance of the two groups to benzyl alcohol, induced and uninduced, is compared.

### RNA extraction

Total RNA was extracted from each group of 20 to 25 flies six hours after the start of the induction protocol using a single-step RNA isolation from cultured cells or tissue protocol (Ausubel, 1994) with some modifications. Flies were ground in liquid nitrogen into a fine powder and transferred to a 1ml dounce homogenizer containing 1ml of denaturing solution (4M guanidinium thiocyanate, 25mM sodiumcitrate, 0.1M 2-mercaptoethanol, 0.5% sarkosyl) and homogenized slowly for 2 minutes. 0.5ml of the homogenate was transferred into a 1.5ml microfugetube. 50 ul of 2M sodium acetate, pH 4, 0.5 ml of water-saturated phenol/

chloroform 5:1 pH 4.5 (Ambion Inc. Austin, TX) plus 0.1 ml of a 49:1 chloroform: isoamyl alcohol mixture (each from Fisher Scientific, Fairlawn, NJ) were added, mixed and incubated for 15 min on ice. The suspension was then centrifuged for 15 minutes at 4°C in a microcentrifuge at maximum speed. The upper aqueous phase containing the RNA was transferred to a fresh 1.5 ml microfuge tube. The RNA was precipitated by adding 1 volume of 100% isopropanol, followed by incubation at –20°C for 20 minutes and centrifugation for 10 minutes at 10,000xg. The dried pellet was then washed in 0.5 ml 75% ethanol, vortexed, and incubated for 10 to 15 minutes at room temperature to extract residual guanidinium thiocyanate. The pellet was centrifuged for 5 min at 10,000xg and the supernatant discarded. The pellet was air-dried for 5-10 minutes and resuspended in 0.2 ml DNase buffer, 3 ul of Superase-In RNase inhibitor and 2 ul of RNase free DNase I (Ambion Inc. Austin, TX) and incubated for 30 minutes at 37°C. Subsequently, the sample was extracted with phenol/ chloroform as before followed by an ethanol precipitation. The pellet was then resuspended in 0.1 ml of DEPC/$H_2O$ and stored at -80°C. RNA quality was determined by electrophoresis in a 1% agarose gel and quantified 260/280 absorption using a nanodrop.

**Quantification of UAS-*slowpoke* transgene induction**

The induction of the *slowpoke* cDNA under the control of the UAS promoter was done using a real-time RT-PCR based assay. The lower

primer was specific to SV40 polyadenylation site found in the transgene cDNA (ATCAGTTGGCAGGTTGGAACGATG) was used in both the reverse transcription and PCR amplification and the upper primer (GATTACGACCATAACTTGCGTGCC) was specific to a region of *slowpoke* included in the same cDNA. The abundance of slowpoke expression after induction was determined by quantifying abundance of a transgenic *slowpoke* expression relative to a message that reflects the total amount of RNA, *Cyclophilin1* using syber green real-time RT-PCR assay. First-strand cDNA was synthesized from 50 ng of total RNA, primed with 200 nM of gene specific lower primers targeting *SV40* and *Cyclophilin1*, using Superscript II reverse transcriptase (Life Technologies). Each reaction was performed in triplicate from independent RNA samples. Six additional reactions were performed from a dilution series of RNA concentrations (0ng, 25ng, 50ng, 100ng, 200ng and 400ng) produced from the control RNA sample to create a standard curve. The standard curve is used for quantification purposes and to prove the linearity of the assay. The first strand synthesis was then diluted to 1/5th the original concentration and one ul was added to 0.5X Power SYBR® Green PCR Master Mix containing the relevant primers. These reactions were conducted in ABI Prism®7700 Sequence Detection System (AppliedBiosystems) thermocycler set to 2 minutes at 95°C followed by 50 cycles of a 30 second denaturing step at 95°C, a 30 second annealing step at 60°C and a 30 second extension step at 72°C. Data was collected

at every cycle during the annealing step. Each PCR was performed in triplicate and averaged. mRNA abundance was extrapolated using the standard curve method. Significance was calculated using the Student's t-Test.

**Quantification of resistance to BA**

Induced and uninduced sex matched flies were exposed to benzyl alcohol coated vials for fifteen minutes. Flies were allowed to recover in clean, benzyl alcohol free, clear glass, vials, as a camera took images of their recovery every 10 seconds.

**Behavioral analysis**

Behavioral recovery was quantified from images taken of the animals recovering, at one frame every ten seconds. Flies are normally negatively geotactic; this behavior ceases while they are sedated. Automated image processing software is used to detect when the flies recover from sedation and return to climbing the walls of their vials [71]. Briefly, the software subtracts images of each vial from the image where all flies are sedated. This resulting subtracted image is void of background and only contains white flies which have recovered and begun climbing. The number of non-black pixels are then counted to generate a quantity that represents the number of flies recovered and returned to negative geotaxis. For each vial, the value at each time point is normalized to a value that represents

complete recovery, giving a percent recovery curve. The percent recovery of each vial within a population is then averaged as a function of time, and plotted with error bars describing the standard error of the mean (SEM). A left-ward shift in the recovery graph indicates resistance to the sedative affects of benzyl alcohol. A statistically significant difference between the two curves is determined using logrank analysis. The animals being tested are treated with BA in a vial coated with BA in a serial fashion with a 30 second delay between each vial. These animals are exposed to benzyl alcohol for 15 minutes, at which point they are transferred to clean fresh vials. The flies are left to recover as a camera acquires images every 10 seconds. These images are then run a program that analyzes the recovery of these animals. Recovery is presented as a percent recovery by standardizing the number of pixels to maximum number of pixels.

## Results

### UAS-*slowpoke* induction is temperature dependent

In order to identify specific neuroanatomical sites of the fly brain which mediate benzyl alcohol resistance, I utilized the temperature-sensitive properties of the Gal4/UAS system to induce *slowpoke* expression in sub-populations of neurons. To circumvent behavioral differences associated with genetic background I only compare induced animals to uninduced animals of the same genotype. Because a loss of a phenotype can be a reflection of the general health of the animals, as opposed to the activity of a specific network I screen for the appearance of a phenotype as opposed to a loss of one.

To induce *slowpoke* expression in specific parts of the nervous system, I incubated animals heterozygous for the Gal4 driver and the UAS-*slowpoke* transgene at 30°C for three days, and compared them to animals incubated at 18°C throughout the three day period. The incubation at 30°C induces *slowpoke* expression because the transcription factor Gal4 binds more tightly to the UAS promoter at 30°C than it does at 18°C [35]. In Figure 5.1 I present data generated by real-time reverse transcription PCR of *slowpoke* expression from the UAS-*slowpoke* transgene in four different Gal4 drivers after incubation at 30°C and 18°C. Across all four Gal4/UAS-*slowpoke* lines, a consistent induction of *slowpoke* expression was evident when comparing animals incubated at

30°C (red) to those incubated at 18°C (green). The four different Gal4 lines tested for induction of *slowpoke* express in cholinergic neurons (cha), dopamine and serotonin containing neurons (ddc), and two lines that express in the mushroom bodies (c309 and ok107).

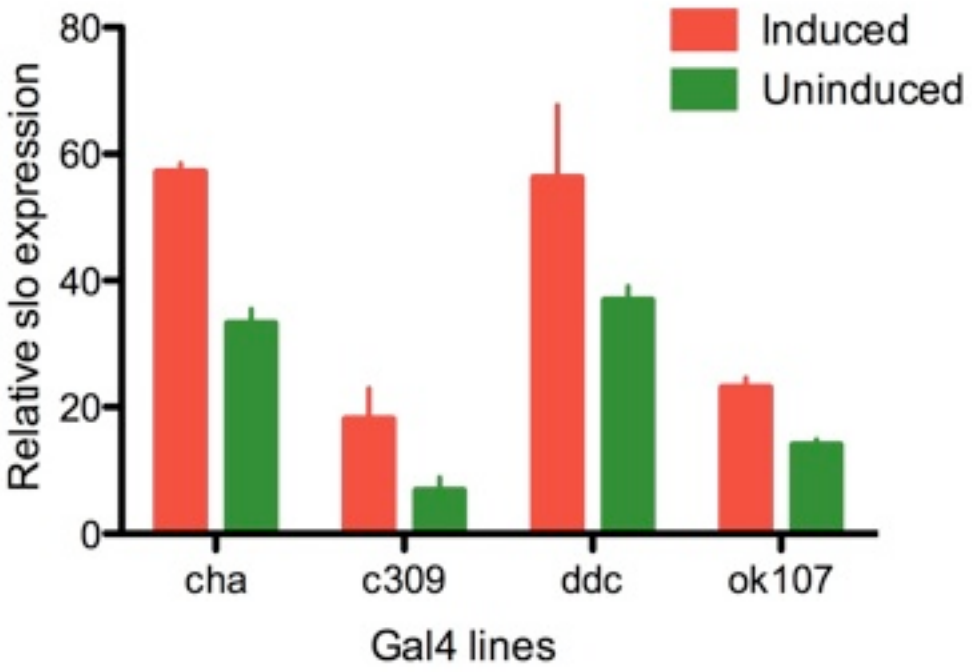



Figure 5.1 Induction of *slowpoke* at elevated temperatures. Induction of *slowpoke* can be achieved by incubating animals heterozygous for the various Gal4 drivers listed on the horizontal axis and the UAS-*slowpoke* transgene at 30°C (red) compared to animals incubated at 18°C (green) which represent the uninduced state.

**Induction of *slowpoke* in the mushroom and ellipsoid bodies causes resistance**

Using the induction protocol described above I tested the effects of *slowpoke* induction by a variety of Gal4 drivers to produce a change in benzyl alcohol sensitivity. Before discussing the details of these experiments it is important to establish that the incubation of wild type flies at 30°C for three days does not alter the recovery from benzyl alcohol sedation (Figure 5.2E). After establishing that this incubation did not alter baseline sensitivity I tested the effects of *slowpoke* induction in the mushroom and ellipsoid bodies on recovery from benzyl alcohol sedation. Of the drivers tested, the ones to produce resistance after *slowpoke* induction were: c041, expressed in the ellipsoid body; 106y, expressed in the ellipsoid and mushroom bodies; and two mushroom body drivers c309 and ok107 (Figure 5.2 A-D). Table 5.1 lists the p-values of these experiments, affect on benzyl alcohol recovery, expression pattern and references to these Gal4 lines. Because *slowpoke* induction within these regions produces resistance, an increase in activity of the mushroom and ellipsoid bodies allows the animals to recover more rapidly from benzyl alcohol sedation, and may represent an endogenous response to benzyl alcohol sedation to produce tolerance.

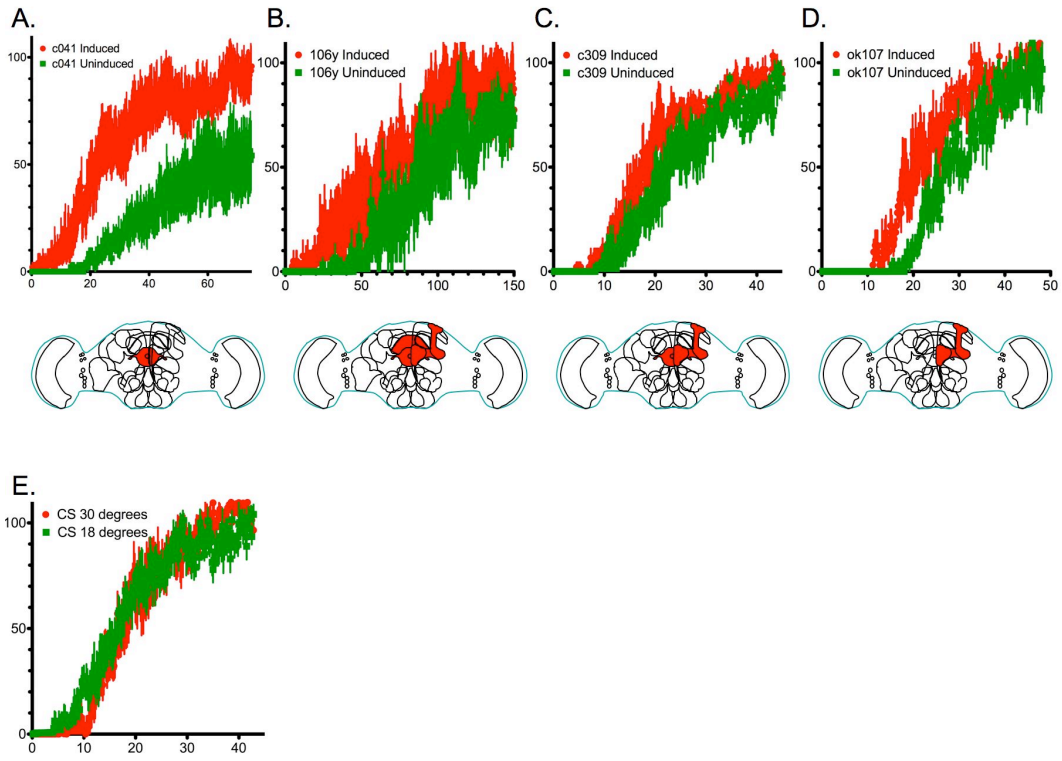



Figure 5.2 Induction of *slowpoke* in the mushroom and ellipsoid bodies causes resistance. A) Induction of *slowpoke* in the ellipsoid body using c041 causes resistance. B) Induction of *slowpoke* in the ellipsoid and mushroom bodies using 106y causes resistance. C) Induction of *slowpoke* in the mushroom bodies using c309 causes resistance. D) Induction of *slowpoke* in the mushroom bodies using ok107 causes resistance. E) Canton S flies are not affected by heat induction protocol.

**Induction of slowpoke in the clock neurons causes sensitization**

Induction of *slowpoke* did not produce resistance in all of the lines tested. I found that induction of *slowpoke* within the Drosophila clock neurons, also known as lateral ventral neurons ($LN_V$s), using two Gal4 drivers pdf and tim causes sensitization to benzyl alcohol sedation (Figure 5.3A and B). Of these two drivers tim Gal4 is more widely expressed but includes the pdf Gal4 expressing cells. I also tested another line, 16y, whose expression pattern is absent in the mushroom bodies but present in the antennal and optic lobes, and found it to also sensitize after induction (Figure 5.3C). These results are summarized in Table 5.1 with columns that indicate the p-value, expression patterns, and references that indicate the regions of expression.

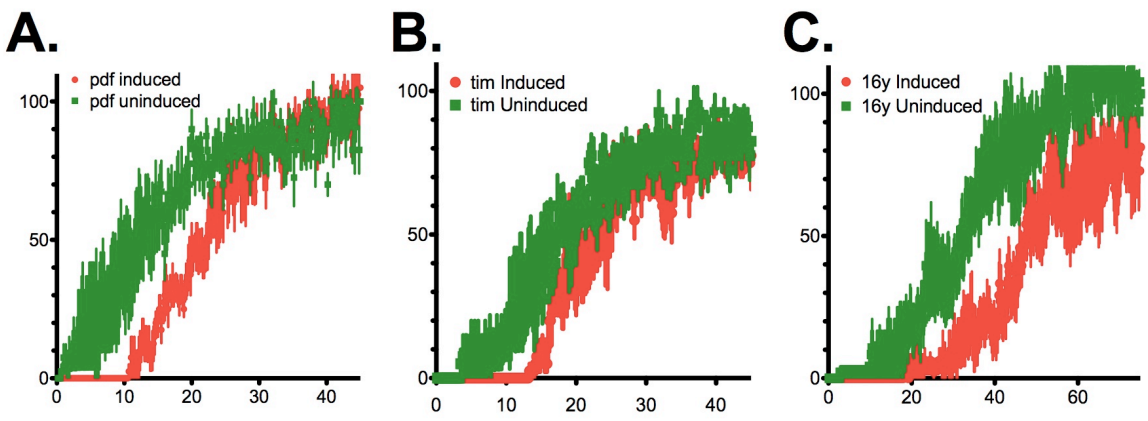



Figure 5.3 Induction of *slowpoke* in three Gal4 lines causes sensitization. A) Induction of *slowpoke* in pdf expressing neurons causes sensitization to benzyl alcohol sedation. B) Induction of *slowpoke* in tim neurons causes sensitization of benzyl alcohol sedation. C) Induction of *slowpoke* in the antennal, optic lobes and $LN_V$s but not the mushroom bodies causes sensitization.

| Lines p<0.05 | p-value | Direction of change | Expression | Reference |
|---|---|---|---|---|
| c041 | <.001 | Resistance | Ellipsoid body | fly-trap.org |
| 106y | <0.009 | Resistance | Mushroom and ellipsoid bodies | fly-trap.org |
| c309 | <0.013 | Resistance | Mushroom bodies | 11 |
| ok107 | <0.02 | Resistance | Mushroom bodies | 9 |
| 16y | <0.002 | Sensitization | Antennal lobes | 11 |
| pdf | <0.002 | Sensitization | lateral ventral neurons | 65 |
| tim | <0.013 | Sensitization | dorsal and ventral lateral neurons | 39 |

Table 5.1 Summary of Gal4 lines that produced significant changes from control. This table summarizes the Gal4 lines that caused a significant change in recovery after induction of UAS-*slowpoke*. The first column is the name of Gal4, the second describes the direction of the change from control, a left-ward shift in the recovery curve is considered resistance, while a right-ward shift is considered sensitization. The third column is a description of the Gal4 expression pattern and the fourth column is a list of references describing the expression patterns in column three.

**Induction of *slowpoke* in several other lines does not change sensitivity.**

I identified seven Gal4 lines which cause a significant change in behavioral recovery after the induction of *slowpoke*, however, there were twelve lines that did not produce a significant change in benzyl alcohol sensitivity. I provide a summary of these lines in Table 5.2, which contains the expression pattern, and reference where available for each of the Gal4 lines tested. All the lines that exhibit a slight but non-significant left-ward shift are mushroom and ellipsoid body drivers. While the two lines that demonstrate a slight but non-significant sensitization are DOPA-decarboxylase (ddc) and a fan shaped body driver (71y) (Figure 5.4).

| Lines p>.05 | p-value | Expression | Reference |
|---|---|---|---|
| 11y | 0.102 | mushroom body | [1] |
| c232 | 0.157 | Ellipsoid Body R3/R4 | [94] |
| 71y | 0.24 | Fan shaped body | fly-trap.org |
| ddc | 0.289 | dopanergic and serotenergic neurons | |
| 103y | 0.318 | mushroom body and Fan shaped | [25] |
| c819 | 0.399 | Ellipsoid body R2/R4 | [73] |
| 238y | 0.444 | mushroom body | [73] |
| c758 | 0.505 | mushroom body optic and antennal lobes | flytrap.org |
| cha | 0.546 | cholinergic | |
| 3741 | 0.554 | unknown | |
| 43y | 0.654 | mushroom body | flytrap.org |
| 10y | 0.893 | Lots of tracts, including mushroom body | flytrap.org |
| 201y | 0.962 | mushroom body and pars intercerebralis | [17] |

Table 5.2 Summary of Gal4 lines that did not exhibit a significant change from control. This table summarizes the Gal4 lines that did not produce a significant change from the uninduced group. The first column provides the name of the Gal4 line, the second column lists the p-values, the third provides expression pattern data compiled from the literature sited in the fourth column.

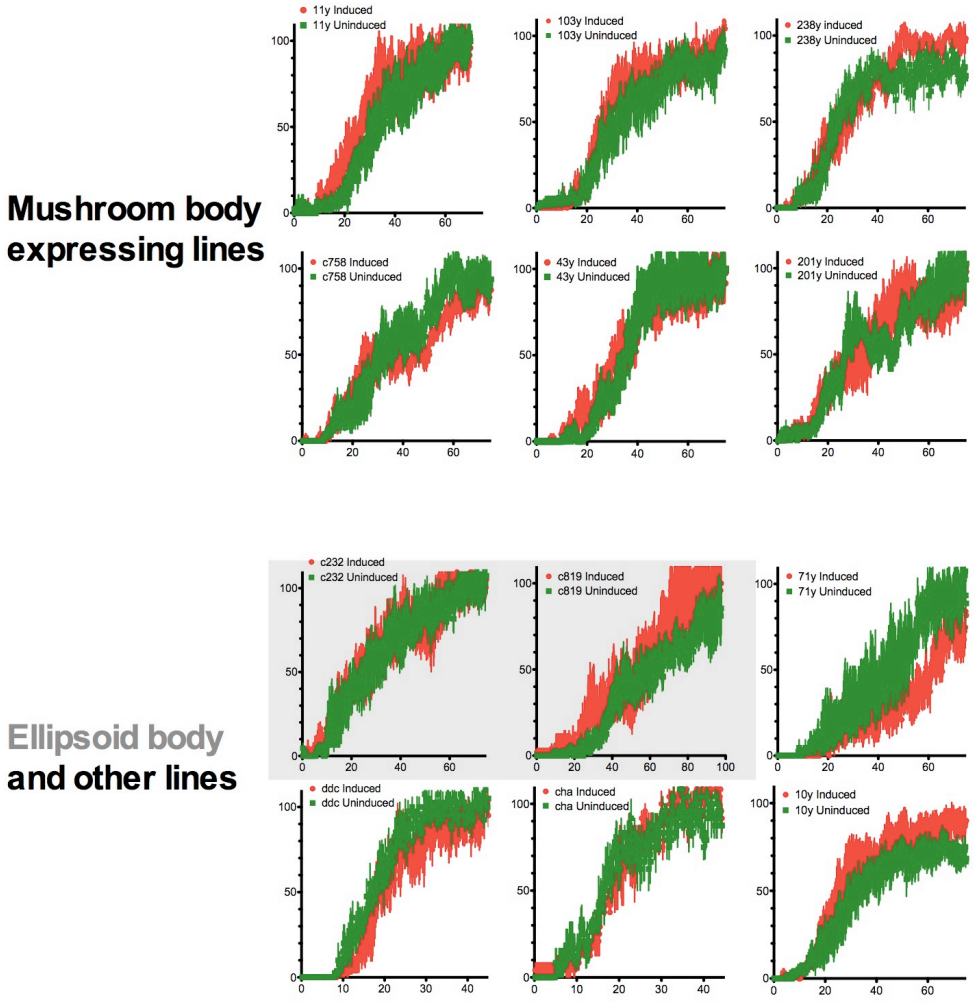



Figure 5.4 Gal4 lines that did not exhibit a significant change from control after induction. This figure contains the recovery plots of sixteen Gal4 lines that did not cause a change in benzyl alcohol sensitivity after the induction of *slowpoke*. The top eight lines are Gal4 line known to express in the mushroom bodies, the bottom eight are Gal4 lines that express in the ellipsoid body (grey shade) and other regions of the brain.

## Discussion

The Gal4 screen conducted to identify the neural substrates that underlie benzyl alcohol resistance and sensitization established the mushroom bodies, the ellipsoid body, and the $LN_V$s clock neurons play a major role in regulating recovery from benzyl alcohol sedation. These structures are responsible for the control and regulation of sleep, arousal and locomotor outputs. The relationship between sleep and anesthesia has been demonstrated in various settings including both mammals and Drosophila.

In human and animal models, where sleep has been extensively studied, several defining characteristics are shared across a wide range of species. Sleep is a recurrent and reversible state of rest with heightened arousal threshold and decreased sensory and motor responsiveness. Sleep is also a state of anabolism where a larger portion of metabolism is devoted to regenerating macromolecules consumed during the awake and active phase. At the level of neuronal signaling, this state is associated with changes in a broad range of nuclei in the human brain. Sleep can be divided into two phases, rapid eye movement (REM) sleep and non-REM (NREM) sleep. Electroencephelogram (EEG) recordings, which measure the gross activity of thousands to millions of neurons, are used refine NREM sleep into phases based on the frequency and amplitude of the

EEG signal. Through the progression NREM sleep the EEG signal evolves from alpha rhythm (8-13 Hz), to theta wave (4-7 Hz), then the appearance of sleep spindles (11-16 Hz), and delta wave (.5-2 Hz). NREM sleep is followed by REM sleep, characterized by a high frequency low amplitude EEG. Sleep also behaves in a homeostatic manner, where sleep deprivation leads to a subsequent increase in NREM and REM sleep. The state of anesthesia is similar to sleep in many respects. Interestingly, anesthesia is able to substitute for the naturally occurring sleep rebound after deprivation [87].

During low levels of anesthesia, EEG profiles demonstrate a reduction in the alpha wave contribution, and as the concentration of the anesthetic increases and the subject enters deeper anesthesia the presence of slow waves increase, and finally as the subject enters deep anesthesia the EEG is transformed to burst suppression pattern [3]. Sleep and anesthesia share common nuclei including those of the thalamus. The thalamic nuclei undergo a decrease in GABA receptor expression in anesthetic tolerant animals [12]. Inhibition of the voltage-activated $K^+$ channel, *Shaker*, in the thalamus also leads to a more rapid recovery from anesthesia.

Recently, Drosophila has been used to study the interaction between sleep and anesthetic sensitivity, resulting in the demonstration that sleep mutants also exhibit differential anesthetic sensitivity. Weber *et. al.* analyzed anesthetic sensitivity of *Shaker* mutants that exhibit a decreased amount of total sleep per day, and found that they require a larger dose of

anesthesia to produce sedation [93]. These discoveries strengthen the notion that sleep and anesthesia share overlapping molecular and neuronal pathways, such that conditions that reduce the amount of sleep also reduce the affects of anesthesia, and *vice versa* where conditions that increase the amount of sleep should also increase the efficacy of anesthetics. In our lab we have found that some mutations that interfere with the circadian control of sleep, *slowpoke* and *period*, also fail to develop tolerance to the anesthetic benzyl alcohol.

In this study, we identified the mushroom bodies and lateral ventral neurons, which are neural sites that regulate sleep maintenance and timing respectively [37,69], to affect recovery from benzyl alcohol sedation. This overlap between the regions involved in the anesthetized state and the centers that control arousal and sleep behaviors demonstrates that a commonality between the two processes also exists in Drosophila at the level of circuitry.

Another brain locus demonstrated to be involved in resistance to benzyl alcohol is the ellipsoid body. This region is part of the central complex and controls locomotor and geotactic behaviors [58]. The ellipsoid body expresses the pdf receptor, thus it is under the regulation of pdf expressing clock neurons. Together with the clock neurons, the ellipsoid body is able to generate a finely tuned circadian control of locomotor behavior. In this assay I score animals as recovered once they return to normal negative geotactic behavior. Thus, the induction of *slowpoke* within

the ellipsoid body can cause benzyl alcohol resistance because the induced population is more active than the control population, and thus begin climbing earlier (Figure 5.5 and 5.6).

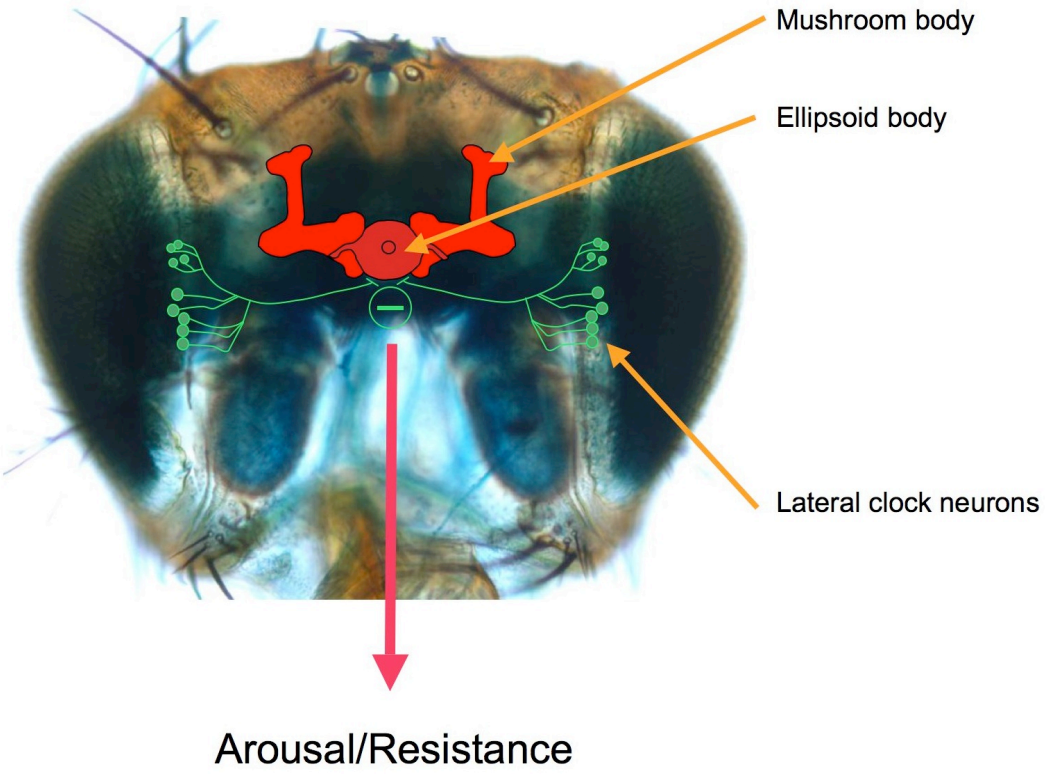



Figure 5.5 Schematic diagram of identified neuronal substrates involved in benzyl alcohol resistance and sensitization. The neuroanotomical sites identified to alter benzyl alcohol sensitivity also regulate arousal. Induction of *slowpoke* within the ellipsoid body and mushroom bodies results in resistance (red), while *slowpoke* induction in the lateral clock neurons causes sensitization (green). Sensitization is caused by inhibition of the ellipsoid body by the lateral clock neurons.

These experiments are based on the assumption that the slowpoke channel is an excitatory molecule, and that induction of *slowpoke* within a brain region should serve to excite that region. The resistance phenotype could arise due to an increase in excitability in the neural substrates that render the animal sedated when inhibited by benzyl alcohol. Thus increasing the excitability of such regions counteracts the sedative affects of benzyl alcohol, and causes the animal to recover more rapidly producing the resistance phenotype. However, not all Gal4 lines caused resistance. Three lines (16y, pdf and tim) produced sensitization to benzyl alcohol after *slowpoke* induction. This observation can be explained by a model where the regions that cause sensitization innervate the regions that cause resistance in a negative or inhibitory manner. Thus exciting those regions increases the inhibition on the regions that cause resistance to produce sensitization.

According to this model, benzyl alcohol inhibition of neuronal structures that mediate arousal renders the animals unconscious. As neuronal signaling is restored in these regions the animal awakens from sedation. An increase in excitability of the arousal centers causes resistance, while an increase in excitability of regions that inhibit the arousal centers causes sensitization. According to the findings discussed here the mushroom and ellipsoid bodies are the structures that mediate arousal, while the clock neurons act to inhibit the structures that mediate arousal (Figure 5.6).

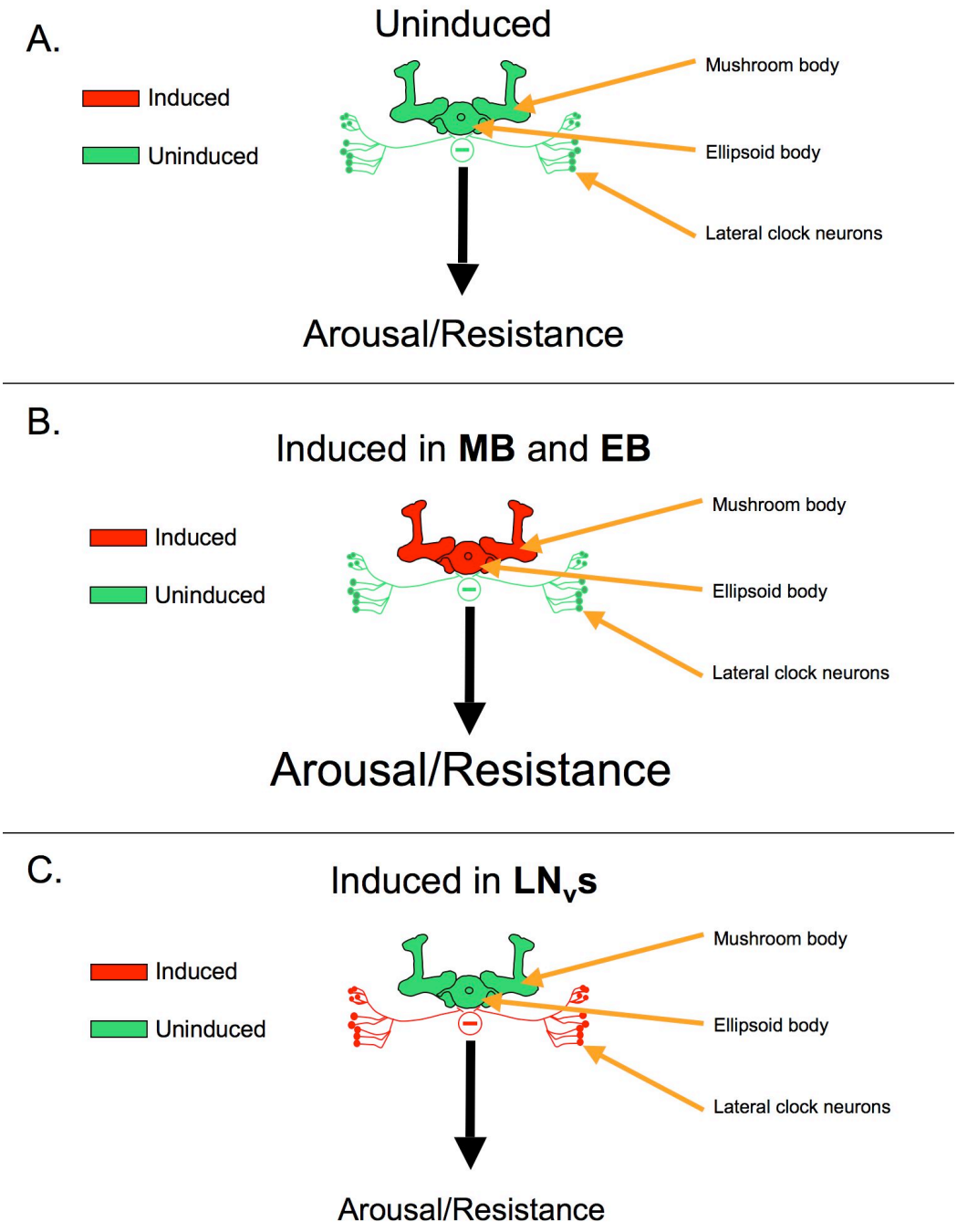



Figure 5.6 Model of neuronal structures that alter arousal and benzyl alcohol sensitivity. A) Represents the activity of the mushroom bodies, ellipsoid body and lateral clock neurons at baseline uninduced conditions. B) Induction of *slowpoke* in the ellipsoid body and mushroom bodies (red) causes an increase in arousal and resistance to the anesthetic benzyl alcohol. C) Induction of *slowpoke* in the lateral clock neurons causes an increase in inhibition of the ellipsoid body, leading to a reduction in arousal and sensitization to benzyl alcohol.

Another possible explanation is that *slowpoke* may also act to reduce excitability in some structures while increasing excitability in others. If this is the case, the clock neurons act to enhance arousal, however, the induction of *slowpoke* reduces their excitability causing a reduction in arousal levels and sensitization to benzyl alcohol sedation.

Even though this study does not directly implicate any brain regions with the development of tolerance, it has identified the circuitry identified to mediate “wake up” behavior. Based on the results of this study we can infer that an upregulation of *slowpoke* function within the mushroom and ellipsoid bodies and the down regulation of *slowpoke* in the clock neurons after benzyl alcohol sedation should cause tolerance. Future experiments that tackle the endogenous structures required for the development of tolerance, could utilize the findings discussed in Chapter 4, where a constitutively active *slowpoke* transgene can rescue the inability of $slo^4$ mutants to develop tolerance. This experiment would simply look for the rescue of tolerance in UAS-*slowpoke* expressing $slo^4$ mutants. Furthermore, *in situ* recording of brain explants, a preparation that conserves neuronal circuitry, could characterize the firing properties of mushroom body, ellipsoid body and clock neurons before and after the induction of tolerance. This experiment would look for changes in neuroexcitability, such as spontaneous or evoked firing frequency induced by benzyl alcohol sedation.

It is notable that not all the mushroom and ellipsoid body expressing Gal4 lines produce resistance to benzyl alcohol. There are at least five possible explanations for the lack of resistance in these mushroom and ellipsoid body expressing lines. The first could be due to low statistical power, where a larger n is required to produce significance in situations where the difference between the induced and uninduced populations is small. A second possibility is that the Gal4 drivers that did not exhibit resistance could express UAS-*slowpoke* to high enough levels such that the further induction of *slowpoke* expression becomes ineffective over such a large baseline. Thirdly it is also possible that the expression of *slowpoke* could be so low that even after induction it cannot alter benzyl alcohol sensitivity. The fourth could be due acute toxicity caused by Gal4 expression which has been demonstrated to cause cell death at high expression levels [74]. It is possible that expression of Gal4 could also render a cell unhealthy enough as to interfere with development of this behavior. Finally it is possible that genetic background affects of the Gal4 lines does not allow for plasticity in this behavior, or that the Gal4 transgene has inserted itself into a region that interferes with the pathways that underlie benzyl alcohol resistance. It is unlikely that the lines demonstrated to produce resistance and sensitization are false positives, as this experiment is based on the gain of a phenotype and not the loss of one.

# Chapter 6: Larval exposure to benzyl alcohol leaves lasting effects in the adult

## Introduction

Metamorphosis is a very active period where neurodegeneration, generation, pruning and remodeling are all occurring rapidly to create a nervous system capable of performing entirely new tasks. As a holometabolous insect, Drosophila undergoes complete metamorphosis to transition from its juvenile larval form to a mature adult. Holometabolous insects have a distinct larval stage that does not resemble the adult. The dramatic process of metamorphosis involves the histolysis of many tissues and reorganization of the nervous system to suit the lifestyle of the new animal. During metamorphosis, some neurons such as the sensory neurons and most interneurons degenerate and are replaced with new ones from imaginal discs. Other neurons such as motor neurons, can survive into adulthood despite the transient loss of their target muscles and are remodeled to perform new functions in the adult, such as walking and flying [84].

Despite the radical nature of metamorphosis, some larval experiences are retained by the adult and alter its behavior. One such experience in Drosophila is olfactory learning. Larvae were trained to avoid an odor using a Pavlovian conditioning protocol, and after metamorphosis the adults continue to avoid the odor [97]. Furthermore, Tully et al. found that

mutations which interfere with the development of long term memory, also fail to express the avoidance response as adults. Similarly, Blackiston et al. in 2008 used a different holometabolous insect, *Manduca sexta*, and found larval conditioning to result in the memory formation which is retained into adulthood [98]. The survival of a memory trace through metamorphosis means that the neurons that encode this memory in the larval mushroom bodies live through metamorphosis into the adult mushroom bodies. It is unknown whether the components of tolerance also persist through metamorphosis. Here I conduct a pilot experiment to test if exposure to benzyl alcohol during juvenile stages affects benzyl alcohol tolerance and resistance in the adult.

## Methods

### Fly stocks

All flies are Canton S wild type flies and were raised on standard cornmeal/molasses/agar medium. Flies were kept in a room at a constant temperature (20°C) and 12:12 hour light:dark cycle.

### Juvenile exposure to benzyl alcohol

Standard standard cornmeal/molasses/agar media was melted on a hot plate, and benzyl alcohol was added as to create a 0.2% concentration by volume. The benzyl alcohol containing media was then allowed to solidify in a cylindrical container, and upon cooling to room temperature flies were transfered into the container and the container was covered with wedding veil. Flies laid eggs in the media for five days, at which point they were discarded. The larvae hatched, then went through three molts. As they began to climb the sides of the container in search of a location to pupate they were harvested and transfered to a benzyl alcohol free vial to pupate and metamorphose. After eclosion, the flies were separated into groups of ten females and tested for resistance and tolerance to benzyl alcohol.

### Coating vials with benzyl alcohol

Clear glass vials (30 ml) with a spherical bottom were coated with either 200 uL of a 0.4% benzyl alcohol solution to test for resistance or

200 uL of a .6% benzyl alcohol solution to test for tolerance. These tubes were rotated continuously at room temperature for 20 minutes to allow the volatile acetone solvent (vapor pressure at 20 °C is 185.6 mm Hg) to evaporate, leaving an even coating of the non-volatile benzyl alcohol (vapor pressure at 20 °C is .07 mm Hg) behind.

**Tolerance and resistance assays**

To test for resistance the recovery was monitored after the first exposure, where six groups of ten age matched benzyl alcohol raised female flies were incubated in benzyl alcohol coated vials for fifteen minutes, and compared to matched control groups. To test for the development of tolerance, animals raised on benzyl alcohol were sedated with benzyl alcohol as adults and compared their recovery from a second sedation was compared to animals raised on benzyl alcohol however were not sedated with benzyl alcohol as adults.

**Behavioral analysis**

Behavioral recovery was quantified from images taken of the animals recovering, at one frame every ten seconds. Flies are normally negatively geotactic; this behavior ceases while they are sedated. Automated image processing software is used to detect when the flies recover from sedation and return to climbing the walls of their vials [71]. Briefly, the software subtracts images of each vial from the image where all flies are sedated. This resulting subtracted image is void of background and only contains white flies which have recovered and begun climbing. The number of non-

black pixels are then counted to generate a quantity that represents the number of flies recovered and returned to negative geotaxis. For each vial, the value at each time point is normalized to a value that represents complete recovery, giving a percent recovery curve. The percent recovery of each vial within a population is then averaged as a function of time, and plotted with error bars describing the standard error of the mean (SEM). A left-ward shift in the recovery graph indicates the presence of tolerance to benzyl alcohol. A statistically significant difference between the two curves is determined using logrank analysis.

## Results

### Larval exposure to benzyl alcohol causes resistance in adults

To demonstrate that a component of benzyl alcohol tolerance can survive through metamorphosis, we tested the tolerance of adult raised on benzyl alcohol containing media. We found that when wild type animals develop in 0.2% benzyl alcohol containing food and are transfered to a benzyl alcohol free environment to metamorphose they are resistant to benzyl alcohol as adults (Figure 6.1A, light orange) when compared to control animals that developed in the absence of benzyl alcohol (Figure 6.1A, green).

### Larval exposure to benzyl alcohol does not prevent rapid tolerance

Upon testing the animals that developed on benzyl alcohol containing media for their ability to further acquire rapid tolerance, we found that animals raised on 0.2% benzyl alcohol were tolerant to the second sedation (Figure 6.1B, crimson) compared to the first (Figure 6.1 B, light orange).

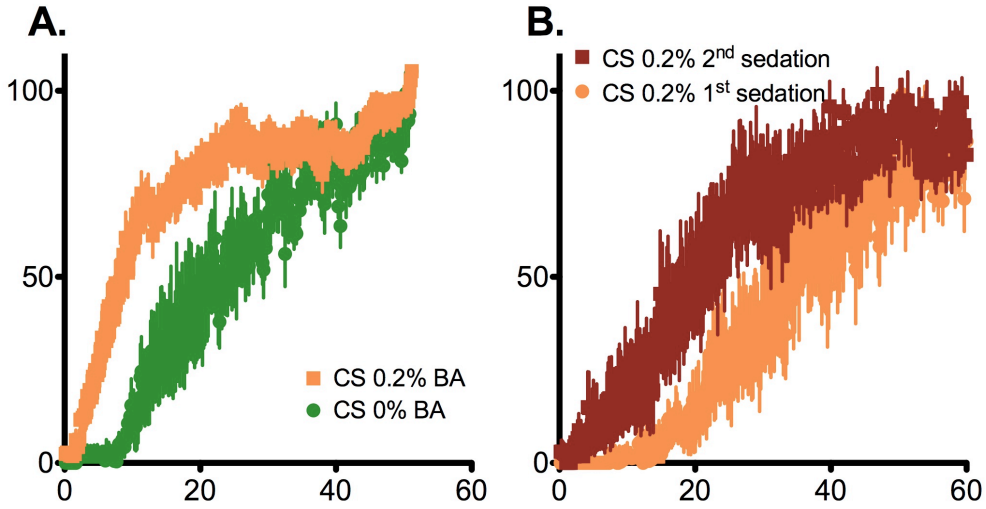



Figure 6.1 Larval exposure to benzyl alcohol causes resistance and does not prevent rapid tolerance in adults. A. is a recovery of adult flies raised on 0.2% benzyl alcohol containing food as larvae from a single sedation (light orange) compared to flies that were raised on standard media that did not contain benzyl alcohol (green). B is a recovery curve of animals that developed on 0.2% benzyl alcohol containing media recovering from their first sedation (light orange) compared to animals recovering from their second sedation (crimson).

## Discussion

The experiments discussed previous chapters of this thesis have been limited to the mechanisms of drug tolerance that occur during the adult stage. This stage is the ultimate stage of development, where the animal does not undergo any major changes as compared to the remodeling and reorganizational events that take place during metamorphosis. Here I ask if benzyl alcohol exposure during early development leaves a lasting effect that survives into adulthood.

In this set of experiments, we explored the influence of larval exposure to benzyl alcohol on the anesthetic properties of benzyl alcohol in the adult. We found that larval exposure to benzyl alcohol causes tolerance to benzyl alcohol administration in the adult. This result signifies the persistence of a larval experience through metamorphosis. I have provided a figure that illustrates the complexity and scale of neuronal remodeling that occurs as the animal passes through the various stages of its life cycle. I chose to provide images of the mushroom bodies during the course of development as they were identified in the screen discussed in Chapter 5 to exhibit anesthetic relevant properties. These images were compiled from work conducted by Lee et. al. published in 1999 [50].

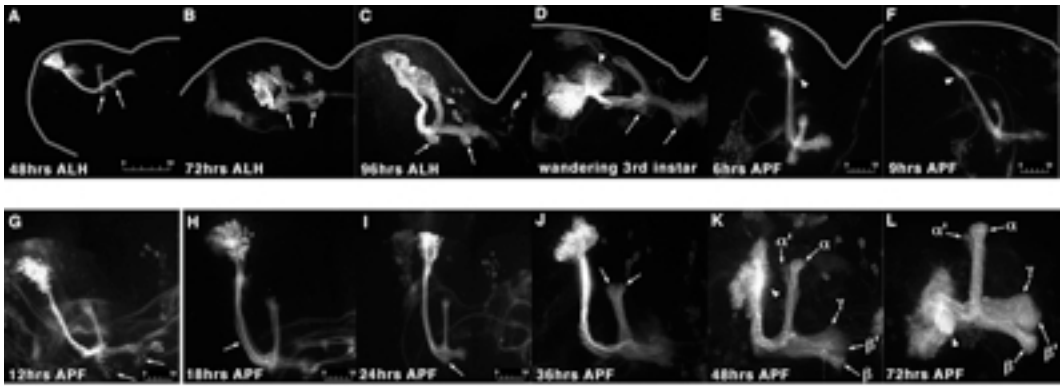



Figure 6.2 Development of the Drosophila mushroom bodies. All images are of a single half of the mushroom body. A-D are of the four points after larval hatching (ALH). E-I are taken during metamorphosis, and J-L are taken during late pupal formation. Notice the extensive dendritic degeneration occurring during early metamorphosis (arrow heads in D-F), which are sprouted in k and L (arrow heads). Compiled from Lee *et al.* (1999).

One significant drawback to this experiment arises from the potential artificial selection for innate benzyl alcohol resistance. There are at least two traits which could select for benzyl alcohol resistance in this experimental paradigm. The first is derived from the observation that females prefer to lay eggs in media which they find suitable, in this case differences between a population derived from eggs deposited on media containing benzyl alcohol to those laid on regular media maybe a reflection of a maternal behavior linked to innate benzyl alcohol resistance, and is subsequently inherited by her offspring. Another possibility for selection could occur during embryonic and larval development. In this case animals predisposed to benzyl alcohol resistance have a larger chance of survival on benzyl alcohol containing media, and thus the resistant phenotype witnessed in the adult would be a reflection of innate resistance as opposed to acquired benzyl alcohol resistance. Future experiments could account for variability in maternal egg laying behavior and innate benzyl alcohol resistance by transferring a known number of embryos from regular media to media containing 0.2% benzyl alcohol and monitoring the number of larvae collected. In this manner maternal behavior is consistent between both populations, and embryonic and larval lethality can be monitored.

If juvenile benzyl alcohol exposures proves to yield acquired benzyl alcohol tolerance it may involve cellular components not utilized during the

acquisition of rapid tolerance. Future experiments designed to identify the components of this form of tolerance should include mutant analysis. The most informative mutations would be the ones that can distinguish between rapid tolerance and tolerance that results due to larval exposure. For example a mutant that fails to acquire rapid tolerance but can develop tolerance that survives through metamorphosis, would indicate the presence of an analogous mechanism unique to larval benzyl alcohol exposure not activated by the adult.

The other observation discussed in this chapter is that animals raised on benzyl alcohol containing food still retain the capacity to develop rapid tolerance. The finding that larval exposure to benzyl alcohol does not occlude the development of rapid tolerance suggests that, either they have two distinct origins, or that they share the same mechanism but rapid tolerance was not saturated by the level of exposure during development. Based on the assumption that juvenile drug exposure produces differing effects than adult drug exposure, one would expect a parallel path to tolerance. This path may include the activation of epigenetic changes, and metabolic and neuronal pathways not activated by adult onset benzyl alcohol tolerance. A comparative micro-array experiment that compares the epigenetic changes caused by larval exposure to benzyl alcohol to adult onset changes would be informative in the study of the long term affects of juvenile drug exposure.

Unlike rapid tolerance this form of tolerance may involve a pharmacokinetic component where metabolic pathways are activated in the larvae due to benzyl alcohol exposure, reducing benzyl alcohol absorption and increasing clearance in the adult. To test this I would use gas chramatography to assay for benzyl alcohol levels after sedation where I compare benzyl alcohol metabolism after sedation between animals raised on 0.2% benzyl alcohol and control animals. If benzyl alcohol concentrations vary between these two populations in a manner which accounts for the observed behavioral differences then it must have pharmacokinetic origins.

# Chapter 7: Summary and Conclusion

Neuronal output that leads to a change in behavior emerges from tightly regulated cellular and molecular events that control neuronal excitability. The nervous system must strike a balance between over-activity and under-activity to maintain normal function. A shift in equilibrium to a more hyperactive state reduces seizure threshold; this governs the upper limit in functional excitability [7]. Meanwhile, a depression in neuronal activity can eventually cause somnolence and coma, defining a lower limit to neuronal excitability. In order for the nervous system to properly perform various tasks, composite networks and neurons must maintain equilibrium within these two limits. We use the anesthetic benzyl alcohol to suppress neuronal excitability, and behavioral plasticity as a reflection of adaptive changes in neuroexcitability.

Anesthesia causes neuronal depression, and tolerance to anesthesia arises from an increase in neural excitability. In this thesis, I explore several aspects of tolerance to the anesthetic benzyl alcohol. I demonstrate that rapid tolerance to benzyl alcohol is a pharmacodynamic, cell autonomous process that requires the presence of a drug, as resistance cannot be induced by a drug-free reduction in neuronal signaling. Beyond previously described transcriptional mechanisms I demonstrate a post-translational role of the $Ca2^{+}$-activated $K^{+}$-channel, slowpoke.

I also demonstrate that the development of tolerance requires proper function of the synaptic vesicle fusion and recycling machinery. Furthermore, I found that not all brain regions are equivalent in mediating benzyl alcohol resistance. Activating some regions such as the mushroom and ellipsoid bodies causes resistance while activating other regions, such as the clock neurons, causes sensitization.

I identify a form of tolerance that is induced by juvenile anesthetic exposure. This form of tolerance most likely depends on the survival of neuronal structures through metamorphosis to allow for the persistence of tolerance into the adult. This form of tolerance may have origins distinct from rapid tolerance as it does not occlude the development of rapid tolerance.

The implications of this study are not only limited to aspects of neuronal plasticity that underlie the development of tolerance. They also provide insight to conditions associated with tolerance such as withdrawal and addiction.

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# Vita

Yazan Mahmoud Al-Hasan was born on July 7, 1979 in Amman, Jordan. The son of Mahmoud Musa Al-Hasan and Nasima Abd Al Kader Hamdi, he is the eldest of four with one brother, Motaz, and two sisters, Lama and Dana. He was raised in Dhahran, Saudi Arabia and attended St. George's School in Newport, Rhode Island from 1994 till 1997. In 1997 he entered the University of Texas at Austin to obtain Bachelor of Science in Molecular Biology in 2001. As an undergraduate he began to conduct experiments in the laboratory of Dr. Nigel Atkinson. He then entered the Neuroscience program at the University of Texas at Austin in 2002 and continued his studies of anesthetic tolerance in the laboratory of Dr. Nigel Atkinson. He was also the recipient of the Pre-emptive Graduate Student Fellowship in 2002.

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